Thomas Van Hecke

Formation of oxidation products and *N*-nitroso-compounds during gastrointestinal digestion of fresh and processed meat

Thesis submitted in fulfilment of the requirements

for the degree of Doctor (Ph.D.) in Applied Biological Sciences

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Dutch translation of the title:

Vorming van oxidatieproducten en *N*-nitroso-verbindingen tijdens gastro-intestinale vertering van vers en bewerkt vlees.

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LIST OF ABBREVATIONS

ALA: α-linolenic acid

ATNC: apparent total nitroso-compounds

CFU: colony forming units

CRC: colorectal cancer

FA: fatty acids

FI: fecal inoculum

HCA: Heterocyclic amines

4-HNE: 4-hydroxy-2-nonenal

LA: linoleic acid

MDA: malondialdehyde

MUFA: monounsaturated fatty acids

LC *n*-3 PUFA: long chain *n*-3 polyunsaturated fatty acids

LC *n*-6 PUFA: long chain *n*-6 polyunsaturated fatty acids

·NO: nitric oxide

NOCs: *N*-nitroso-compounds

O^6-CMG: O^6-carboxymethylguanine

O^6-MG: O^6-methylguanine

PAH: Polycyclic aromatic hydrocarbons

PCC: protein carbonyl compounds

ROS: reactive oxygen species

SFA: saturated fatty acids

TBARS: thiobarbituric acid reactive substances

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Introduction

Already in 1900, surgeon William Banks suggested that the increase of cancer in his time was related to the more general consumption of "butcher's meat" (Haddon, 1900). More than 115 years later, a vast amount of epidemiologic studies consistently show a positive association between high red and/or processed meat consumption and a wide range of diseases, among which colorectal cancer (CRC) (Meta-analyses by Chan et al., 2011; Aune et al., 2013; Xu et al., 2013), coronary heart disease (Meta-analyses by Micha et al., 2010; 2012) and diabetes (Meta-analyses by Micha et al., 2010; Pan et al., 2011). In 2007, the World Cancer Research Fund advised to limit red meat consumption and to avoid processed meat, which was later supported by the Superior Health Council of Belgium (2013). Recently, the 'International Agency for Research on Cancer' (Bouvard et al., 2015) classified processed meat as 'carcinogenic to humans' (Group 1) and red meat as 'probably carcinogenic to humans' (Group 2A).

The term 'red meat' in this epidemiological context does not refer to the degree of doneness of the meat upon cooking, but refers to meats with a high concentration of haem-Fe. E.g. white meat such as chicken has low haem-Fe contents (0.12-0.29 mg/100g), while both pork (0.20-0.32 mg/100g) and beef (1.68-2.11 mg/100g) are considered red meat, even though pork has considerately lower haem-Fe concentrations compared to beef (Lombardi-Boccia et al., 2002). A precise definition of 'processed meat' is lacking, it is usually described as meats preserved by smoking, curing or salting or by the addition of chemical preservatives (Demeyer et al., 2008). Nitrite salt is widely used as a curing agent in meat products to inhibit outgrowth of *Clostridium botulinum*, spoilage, oxidative rancidity and to obtain a desired red meat color.

The responsible mechanisms explaining the association between high red (processed) meat consumption and various chronic diseases are still a matter of debate, even though several plausible hypotheses have been formulated over the years. Diseases associated with high red (processed) meat consumption have one important condition in common; oxidative stress is suggested to exert a central role in their etiology and/or progression (Corpet, 2011; Chen & Keaney, 2012; Rains & Jain, 2011). Moreover, the occurrence of these diseases is often inter-related, indicating a common element in their etiology (Ceriello & Motz, 2004; De Bruijn et al., 2013; Lee et al., 2013 Peters et al., 2014). Next to oxidative stress, the formation of genotoxic *N*-nitroso-compounds (NOCs) during red (processed) meat digestion is suggested to contribute to the association between red (processed) meat consumption and CRC (Cross et al., 2003; Lewin et al., 2006).

Meat is a complex medium containing fat, proteins, free and bound iron, which are all compounds participating in oxidation processes. In the case of processed meats, the composition is even more complex and variable due to possible mixing with non-meat ingredients and the use of nitrite, salt and/or other additives. The kind of processing (e.g. cooking, fermentation…) and the processing conditions also affect the susceptibility to oxidation processes. During the self-maintaining Fenton reaction, hydrogen peroxide (H_2O_2) catalyzes the oxidation of Fe^{2+} to Fe^{3+} (1) and its reduction back to Fe^{2+} (2), hereby producing a hydroxyl radical (HO·) and a hydroperoxyl radical (HOO·):

$$(1)\ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-}$$

$$(2)\ Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO^{\bullet} + H^{+}$$

These formed Reactive Oxygen Species (ROS) are highly unstable and initiate a chain of oxidative reactions. Oxidative modifications to *n*-3 and *n*-6 polyunsaturated fatty acids (PUFAs) result in the formation of cyto- and genotoxic lipid oxidation products (LOP), among which malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Guéraud et al., 2010). Formed ROS and LOP also contribute to the formation of protein oxidation products (Estévez, 2011).

During gastrointestinal digestion, meat is exposed to the specific conditions in the different compartments of the digestive tract. Successively, meat is exposed to saliva in the mouth, acid gastric juice in the stomach, emulsifying pancreatic and bile juice in the small intestine and anaerobic fermentation by microbiota in the large intestine. Therefore, the environment in every compartment of the digestive tract may influence oxidative processes during meat digestion differently. Saliva can exert a dual role in oxidation, whereby saliva of donors exerted either pro-oxidant, antioxidant or no effect, depending on their composition (*e.g.* nitrite, thiocyanate, peroxidase) (Gorelik et al., 2007). Kanner & Lapidot (2001) showed the stomach is an excellent medium to enhance lipid oxidation in heated muscle tissue due to its low pH and its dissolved oxygen. Feeding heated red turkey cutlets to rats increased lipid hydroperoxides and MDA in the stomach content (Gorelik et al., 2008a; Kanner et al., 2012). In the small intestine, secreted lipases and bile salts emulsify the intestinal contents (McClements and Li, 2010), which could affect the oxidation reaction mechanisms since it is well known that these are considerably different among aqueous solutions, bulk fat or emulsions (McClements and Decker, 2000). A high proportion of ingested haem-Fe reaches the colon (Pierre et al., 2008) and could therefore stimulate oxidative reactions in colonic contents. It is described that some *Lactobacillus* strains exert antioxidant behavior by preventing the Fenton reaction (Sun et al., 2010), while other bacterial species such as *Enterococcus faecalis* are able to stimulate the Fenton reaction by producing

extracellular superoxide (Huycke et al., 2002). Therefore, the colonic microbial composition likely also has an influence on oxidation processes. Martin et al. (2015) reported a decrease in fecal TBARS when rats on a 2.5% hemoglobin diet were treated with a cocktail of antibiotics. The authors suggested the reduction of lipid oxidation was attributed due to the suppression of the colonic microbiota. However, an (additional) antioxidant effect of the antibiotics itself cannot be excluded.

In 1996, Bingham et al. proposed that an increased endogenous production of NOCs and its precursors from the consumption of red (processed) meat, but not white meat and fish, may be relevant to the etiology of CRC. In a series of human intervention studies, they demonstrated a dose-responsive increase in fecal excretion of NOCs with red meat intake, which was not observed with vegetable proteins, white meat or an Fe^{2+} supplement, but clearly mimicked by a haem-Fe supplement (Hughes et al., 2001; Bingham et al., 2002; Cross et al., 2003). However, the analytical method used to analyze NOCs failed to differentiate the toxic *N*-nitroso-compounds from the non-toxic compounds such as *S*-nitrosothiols, *O*-nitroso compounds and nitrosyl-Fe. Therefore, the term ATNC (apparent total *N*-nitroso-compounds) was used to describe the substances measured by this technique. In 2007, Kuhnle et al. introduced an adaptation to the method, allowing to distinguish between nitrosyl-Fe and nitrosothiols. Lewin et al. (2006) showed that human volunteers consuming high amounts of red meat had increased fecal ATNC, which correlated with the NOC-derived DNA-adduct O^6-carboxymethylguanine (O^6-CMG) in colonic exfoliates. Rats treated with the *N*-nitrosopeptide *N*-acetyl-*N*'-prolyl-*N*'-nitrosoglycine showed the presence of O^6-CMG in the intact small intestine. This was also observed in HT-29 cells treated with diazoacetate (Lewin et al., 2006). Since the analysis for ATNC includes both toxic and non-toxic compounds, the quantification of O^6-CMG might offer a more specific insight in the formation of genotoxic NOCs.

It is still largely unknown how the different characteristics of meat products and the diet influence the formation of oxidation products and NOCs during digestion. Therefore, this Ph.D. dissertation mainly focuses on the formation of lipid and protein oxidation products and DNA adducts putatively derived from NOC during *in vitro* digestion of various meat products. In addition, strategies to inhibit this formation were investigated by adding reducing compounds during meat digestion. Finally, a rat feeding study was conducted to validate the *in vitro* observations and to study the effect of meat consumption on systemic oxidative stress parameters. An outline of the thesis is shown in Figure I.1.

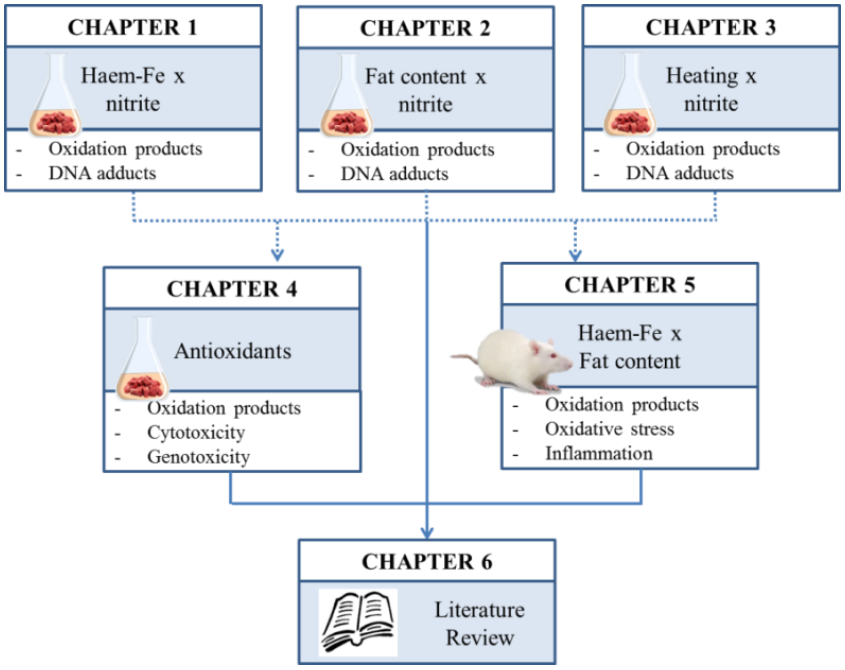

Figure I.1: outline of the Ph.D. thesis including four *in vitro* digestion studies, one rat feeding study and a literature review.

An *in vitro* digestion protocol was developed, specifically for studying oxidation during digestion. At first, experiments focused on the role of several meat-related factors on oxidation and nitrosation during digestion. Among others, meat products differ in their content of haem-Fe, fat and the use of nitrite salt. Furthermore, meat products can be exposed to various heating

treatments. Therefore, different model meat products were produced and digested to investigate the influence of these factors on the formation of oxidation and nitrosation products. More specifically, the interaction of haem-Fe, fat content and heating conditions in combination with nitrite-curing of meat was studied in the first three chapters of this Ph.D.

- **Chapter I – haem-Fe × nitrite**: Nitrite-curing of chicken, pork and beef inhibits oxidation but does not affect NOC-specific DNA adduct formation during *in vitro* digestion.

- **Chapter II – fat content × nitrite**: Fat content and nitrite-curing influence the formation of oxidation products and NOC-specific DNA adducts during *in vitro* digestion of meat.

- **Chapter III – heating × nitrite**: Increased oxidative and nitrosative reactions during digestion could contribute to the association between well-done red meat consumption and colorectal cancer.

Previous research chapters showed that oxidation and nitrosation are increased during the digestion of heated meat products high in haem-Fe and fat content. Subsequently, the potential of various reducing compounds was tested, with the aim to mitigate the formation of oxidation products during the digestion of these model meat products. The results of these experiments are described in Chapter IV.

- **Chapter IV – reducing compounds**: Reducing compounds equivocally influence oxidation during digestion of a high-fat beef product, which promotes cytotoxicity in colorectal carcinoma cell lines.

Previous digestion experiments were all performed under *in vitro* conditions. However, the *in vivo* situation is more complex, e.g. there is no absorption during *in vitro* digestions and the interaction of formed metabolites with various tissues can evidently not be studied. Hence, in a short-term rat feeding trial, we investigated the main factors that stimulate the *in vitro* formation of oxidation products during digestion of heated meat products, namely haem-Fe and fat content. Next to the formation of oxidation products in the gastro-intestinal tract, also systemic oxidative stress and inflammation markers were investigated in Chapter V.

- **Chapter V - haem-Fe × fat content**: Short-term beef consumption promotes systemic oxidative stress, TMAO formation and inflammation in rats, and dietary fat modulates these effects.

Finally, an extensive study of the literature in combination with the data generated during this Ph.D. resulted in a review chapter which specifically focusses on the formation of oxidation products during red (processed) meat digestion, how dietary compounds may modulate this formation and an exploration of mechanisms linking oxidative reactions with the onset and/or progression of chronic diseases. Some additional hypotheses have been formulated including the possible involvement of *Helicobacter* gastritis. Infection with *H. pylori* turns the stomach into a highly oxidizing environment, which could promote the formation of lipid oxidation products during the digestion of red (processed) meat. The possible involvement of *H. pylori* is extensively reviewed in this chapter, and could be an interesting hypothesis to investigate in further research.

- **Chapter VI – review**: The role of red (processed) meat in the diet and *Helicobacter pylori* gastritis in oxidative stress-associated diseases.

Chapter I

Nitrite-curing of chicken, pork and beef inhibits oxidation but does not affect NOC-derivative DNA adduct formation during *in vitro* digestion

Adapted from:

Van Hecke, T., Vanden Bussche, J., Vanhaecke, L., Vossen, E., Van Camp, J., & De Smet, S. (2014). Nitrite Curing of Chicken, Pork, and Beef Inhibits Oxidation but Does Not Affect *N*-Nitroso Compound (NOC)-Specific DNA Adduct Formation during *in Vitro* Digestion. *Journal of Agricultural and Food Chemistry*, 62(8), 1980-1988.

ABSTRACT

Uncured and nitrite-cured chicken, pork and beef were used as low, medium and high sources of haem-Fe respectively, and exposed to an *in vitro* digestion model simulating mouth, stomach, duodenum and colon. With increasing content of iron compounds, up to 25-fold higher concentrations of the toxic lipid oxidation products MDA, 4-HNE and other volatile aldehydes were formed during digestion, together with increased protein carbonyl compounds as measurement of protein oxidation. Nitrite-curing of all meats lowered lipid and protein oxidation to the level of oxidation in uncured chicken. Strongly depending on the individual fecal inoculum, colonic digestion of beef resulted in significantly higher concentrations of the NOC-derivative DNA adduct O^6-CMG compared to chicken and pork, while nitrite-curing had no significant effect. This study confirms previously reported evidence that haem-Fe is involved in the epidemiological association between red meat consumption and CRC, but questions the role of nitrite-curing in this association.

INTRODUCTION

Colorectal cancer is the second most commonly diagnosed cancer in women and the third in men (WCRF, 2007). Different independent meta-analyses of epidemiologic studies demonstrate a significant increased CRC risk associated with a higher consumption of red meat and especially processed meat (Larsson & Wolk, 2006; Chan et al., 2011). Consumption of poultry, which contains lower amounts of haem-Fe than pork and beef has not been associated with CRC and high consumption of fish is associated with a lower risk (Santarelli et al., 2008). In 2007, the World Cancer Research Fund/American Institute for Cancer Research made the recommendation to limit red meat consumption and avoid processed meat as much as possible.

The biochemical mechanisms responsible for these epidemiologic associations have not yet been completely elucidated. Corpet (2011) suggested that haem-Fe in meat exerts a catalytic effect on the endogenous formation of geno- and cytotoxic oxidation products such as MDA and 4-HNE and on the formation of genotoxic NOCs. Several mechanistic studies support a stimulating role for haem-Fe in endogenous oxidation (Pierre et al., 2004; 2006) and nitrosation reactions (Bingham et al., 2002; Cross et al., 2003; Lewin et al., 2006). Both *in vivo* and *in vitro* studies have demonstrated increasing lipid peroxidation during passage through the gastrointestinal system (Hur et al., 2009: Kanner et al., 2001; Gorelik et al., 2008a). Subsequently, DNA can be damaged by binding with previously mentioned genotoxic compounds, e.g. DNA adducts formed with MDA (pyrimido[1,2-α]purine-10(3H)-one-2'-deoxyribose), 4-HNE (e.g. 1,N^6-etheno-2'-deoxyadenosine) and NOCs (e.g. O^6-carboxymethylguanine) (Lewin et al., 2006; Nair et al., 2007). Unlike the role of haem-Fe, the importance of nitrite-curing of meat in this context is not well studied. Nitrite salt is widely used as a curing agent in meat products to inhibit outgrowth of *Clostridium botulinum*, spoilage, oxidative rancidity and to obtain a desired red meat color. The antioxidative mechanism of nitrite in meat was demonstrated by the group of Kanner (1994) which involves antioxidant activity of formed nitric oxide myoglobin, nitric oxide ferrous complexes and *S*-nitrosocystein and inhibition of the Fenton reaction which is responsible for the initiation of oxidation reactions. Furthermore, a stabilizing effect of nitrite was observed on the susceptibility of unsaturated lipids in the membranes to oxidation. In acidic conditions such as present in the stomach, nitrous acid generates dinitrogen trioxide (N_2O_3) and H_2O which is in equilibrium with nitric oxide (·NO) and nitrogen dioxide (·NO_2) (Honikel, 2008). A dual role of ·NO on lipid oxidation was described whereby a 1:1 ratio of ·NO to ROS enhances lipid oxidation while an excess of ·NO inhibits oxidation (Darley-Usmar et al., 1995). Corpet (2011) suggested that nitrite-curing of meat would be an important contributor to NOC-formation. In

aerobic conditions, ·NO reacts with O_2 to form nitrosating species (e.g. N_2O_3) (Espey et al., 2002), which induce the formation of genotoxic NOCs.

The aim of this study was to elucidate the likely mediating effect of nitrite-curing on haem-Fe-induced oxidative- and nitrosative stress during digestion of meat. For this purpose, uncured and nitrite-cured cooked chicken, pork and beef as low, medium and high sources of haem-Fe respectively, were subjected to a multi-compartment *in vitro* digestion model. In the digests, the major toxic lipid oxidation products MDA and 4-HNE and the less toxic simple aldehydes pentanal, hexanal, heptanal, nonanal were determined. During oxidation of proteins, carbonylation is one of the most prominent protein modifications (Estévez, 2011) and hence, measurement of protein carbonyl compounds (PCC) was used to estimate protein oxidation during digestion. The NOC-derivative DNA adduct O^6-CMG was determined in digesta to estimate DNA damage induced by nitrosative stress. O^6-CMG is a far more specific endpoint than the commonly used ATNC because (i) it demonstrates DNA damage caused by alkylating NOCs and (ii) ATNC also include non-carcinogenic nitrosyl-Fe and nitrosothiols next to the carcinogenic NOCs.

MATERIALS AND METHODS

Experimental set-up

Uncured and nitrite-cured cooked chicken, pork and beef were compared before and after mimicked duodenal and colonic digestion. Each incubation run included all meat treatments (6) in quadruplicate, from which 2 duplicates underwent digestion until the duodenum and 2 until the colon step. Each incubation run was performed in triplicate with each time a fecal inoculum (FI) originating from a different human volunteer.

Chemicals

All the digestive enzymes; mucin from porcine stomach type II (M2378), pepsin from porcine gastric mucosa (>250 U/mg solid; P7000), lipase from porcine pancreas type II (10-400 U/mg protein; L3126), pancreatin from porcine pancreas (8 × USP specifications; P7545) and porcine bile extract (B8631) were purchased from Sigma Aldrich (Diegem, Belgium).

Manufacturing of meat samples

Lean meat samples from the *m. Pectoralis profundus* (chicken), *m. Longissimus dorsi* (pork) and *m. Biceps femoris* (beef) were purchased in a local supermarket. The meat was manually chopped into cubes of approximately 1-2 cm^3. To exclude a possible confounding effect of differences in fatty acid composition, 4% subcutaneous pork fat was added to the different lean meat sources. Meat samples with added fat were first minced in a grinder (Omega T-12) equipped with a 10 mm plate, followed by grinding through a 3.5 mm plate. Thereafter, nitrite-curing was applied by adding 20 g 0.6% nitrite salt/kg meat, corresponding to an added concentration of 120 mg nitrite/kg meat, after which the meat samples were manually homogenized. All meat samples were heated in an open glass container in a warm water bath for 15 min after the core temperature had reached 65°C. After manufacturing, all meat samples were homogenized in three 5s bursts using a food processor (Moulinex DP700), vacuum packed and stored at -20°C until the start of the incubation.

Digestive simulations

The *in vitro* digestions consisted of an enzymatic digestion simulating the mouth, stomach, and duodenum gastro-intestinal tract compartments, followed by a simulation of colonic

fermentation (Figure 2.1). For the enzymatic digestion, the protocol described by Versantfoort et al. (2005) was adapted by adding oxidants and antioxidants that are normally present in digestive juices (Table 1.1). Hence, peroxidase (Güven et al., 1996) and $NaNO_2$ (Takahama et al., 2003) were added to the saliva juice, and ascorbic acid (Dabrowska-Ufniarz et al., 2002), H_2O_2 (Nalini et al., 1992) and ferrous iron (Nalini et al., 1992) were added to the gastric juice. Meat samples (4.5 g) were sequentially incubated for 5 minutes with 6 mL saliva, 2 hours with 12 mL gastric juice, and 2 hours with 2 mL bicarbonate buffer (1 M, pH 8.0), 12 mL duodenal juice and 6 mL bile juice. These enzymatic incubations were performed in quadruplicate. After completion, 2 replicates were diluted with 44 mL H_2O to obtain the same solid/liquid ratio as in the colonic digestion (see further), homogenized with an ultraturrax (9500 rpm). While stirring on a magnetic field in the dark, samples were subdivided in 1.3 mL aliquots and stored at -20°C and -80°C pending analysis. The 2 remaining replicates underwent the additional colonic fermentation stage according to Van de Wiele et al. (2005). SHIME (simulation of the human intestinal microbial ecosystem) medium (22 mL) (Molly et al., 1994) and a human fecal inoculum (22 mL) (for preparation see next paragraph) were added to the digesta. The vessels were flushed with N_2 for 30 minutes to obtain an anaerobic environment. Anaerobic conditions in flasks with digesta in the colon phase were confirmed, using resazurin saturated test strips. Subsequently, the vessels were incubated for 72 hours while stirring at 37°C. As for the duodenal samples, colonic digestion samples were homogenized by ultraturrax at 9500 rpm. While stirring on a magnetic field in the dark, samples were subdivided in 1.3 mL aliquots and stored at -20°C and -80°C till analyses. Undigested control samples were obtained in duplicate by homogenizing 4.5 g meat in 82 mL H_2O, mimicking the liquid/solid ratio in the digested samples.

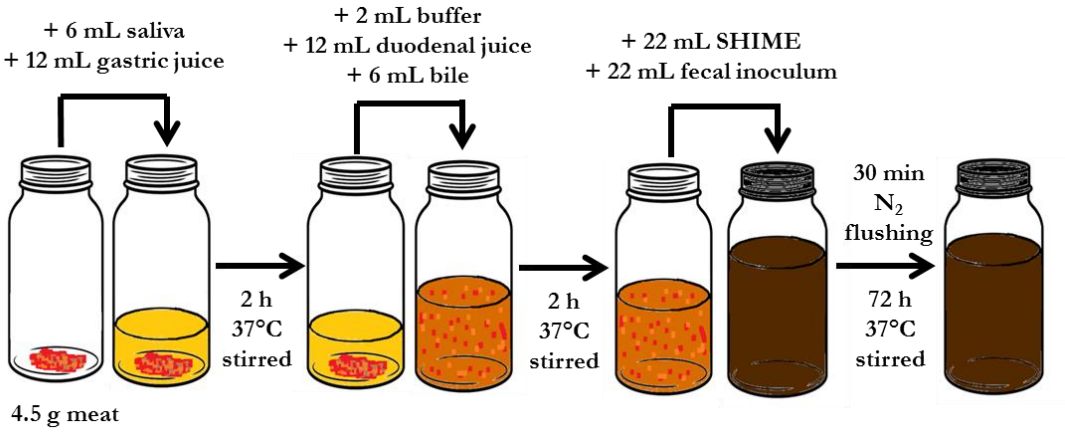

Figure 2.1: *In vitro* digestion model simulating the enzymatic digestion in the mouth, stomach, and duodenum, followed by a simulation of colonic fermentation.

Preparation of human fecal inoculum

Fresh fecal material was collected from 3 volunteers without known gastro-intestinal diseases and without intake of antibiotics for at least 6 months. All volunteers were male, non-vegetarians on a Western diet, and aged 49, 26 and 38 years, respectively. Fresh fecal material was diluted in pre-heated PBS solution (1/4; w/v), to which sodium thioglycolate (1 g/L) was added as a reducing agent. The fecal slurry was filtered by a 1 mm metal sieve to remove particulate matter. Afterwards, the inocula were stored at -80°C on glycerol stock (20%) in different aliquots. Before use in the colonic fermentation phase, the bacterial inoculum was cultured during 24 hours at 37°C to obtain the necessary microbiotic culture. For this purpose, fecal inoculum was diluted with BHI broth (37 g/L Brain Heart Infusion and 0.5 g/L cysteine) at 1/9 ratio. Subsequently, anaerobic conditions in the flask were reached by flushing the headspace with N_2 during 1 hour.

Table 1.1: Composition of digestive juices (1 L) used for *in vitro* incubation of meat samples.

	ENZYMATIC DIGESTION[1]								FERMENTATION	
	Mouth		Stomach		Duodenum				Colon	
	Saliva (pH 6.8)		Gastric juice (pH 1.3)		Duodenal juice (pH 8.1)		Bile (pH 8.2)		SHIME medium[2] (pH 7.0)	Cultured Bacterial inoculum*
Inorganic Sol.	0.90 g KCl		2.75 g NaCl		7.01 g NaCl		5.26 g NaCl		6.8 g KH_2PO_4	0.51 g NaCl
	0.20 g KSCN		0.27 g NaH_2PO_4		3.39 g $NaHCO_3$		5.79 g $NaHCO_3$		8.8 g K_2HPO_4	0.01 g KCl
	0.90 g NaH_2PO_4		0.82 g KCl		0.08 g KH_2PO_4		0.38 g KCl			0.23 g $Na_2HPO_4.12H_2O$
	0.57 g $NaSO_4$		0.40 g $CaCl_2.2H_2O$		0.56 g KCl		0.15 mL HCl 37%			0.02 g KH_2PO_4
	0.30 g NaCl		0.31 g NH_4Cl		0.05 g $MgCl_2$					
	1.69 g $NaHCO_3$		6.50 mL HCl 37%		0.18 mL HCl 37%					
Organic Sol.	0.20 g Urea		0.09 g Urea		0.10 g Urea		0.25 g Urea		1.0 g Arabinogalactan	0.06 g $C_2H_3NaO_2S$
	11.5 mg Uric acid		0.02 g Glucuronic acid		1.00 g BSA				3.0 g Yeast extract	33.3 g Brain Heart Infusion
	25.0 mg Mucin		0.65 g Glucose		9.00 g Pancreatin		1.80 g BSA		0.4 g Glucose	0.45 g L-Cystein-HCl
	2.50 IU Peroxidase[3]		0.33 g Glucosamine-HCl		1.50 g Lipase		30.0 g Bile		0.5 g L-Cystein-HCl	16.0 mL Fecal matter
			17.6 mg Ascorbic acid[5]						4.0 g Mucin	20.0 mL Glycerol
			1.00 g BSA						2.0 g Pectin	
			2.50 g Pepsin						1.0 g Pepton	
			3.00 g Mucin						1.0 g Xylan	
									4.0 g Potato starch	
Add	6.90 mg $NaNO_2$[4]		10.0 µl H_2O_2 (30%)[6]		0.200 g $CaCl_2.2H_2O$		0.222 g $CaCl_2.2H_2O$			
			11.2 mg $FeSO_4.7H_2O$[7]							

ENZYMATIC DIGESTION[1]: based on Versantfoort et al. (2005) unless otherwise indicated; SHIME medium[2]: Molly et al., 1994; Peroxidase[3]: Güven et al., 1996; $NaNO_2$[4]: Takahama et al., 2003; Ascorbic acid[5]: Dabrowska-Ufniarz et al., 2002; H_2O_2[6]: Nalini et al., 1992; $FeSO_4$[7]: Nalini et al., 1992.

* Bacterial inoculum was cultured under anaerobic conditions for 24 hours at 37°C and used immediately in the fermentation procedure.

Chemical composition of the meat samples

Meat samples were analyzed for dry matter, crude protein and crude fat content according to the ISO 1442–1973, ISO 937–1978 and ISO 1444–1973 methods, respectively. Lipids were extracted using chloroform/methanol (2/1; v/v) (Folch et al., 1957) and subsequently, fatty acids (FA) were analyzed as described by Raes et al. (2001). Briefly, FA were methylated and analyzed by gas chromatography (HP6890, Brussels, Belgium) using a CP-Sil88 column for fatty acid methyl esters (FAME; 100 m × 0.25 mm × 0.25 μm; Chrompack, the Netherlands). Peaks were identified, based on their retention times corresponding with standards (NuChek Prep. Inc., Sigma, Bornem, Belgium). Nonadecanoic acid (C19:0) was used as an internal standard to quantify the individual and total FA. The fatty acid profiles were expressed in g/100g FAME. Residual nitrite concentrations were measured colorimetrically at 538 nm after diazotization with sulfanilamide and *N*-(1-naphtyl)-ethylenediamine dihydrochloride (ISO 2918-1975). Nitrite concentrations were calculated based on a standard curve obtained with sodium nitrite and expressed as mg nitrite/kg meat. Hematin was determined colorimetrically by the method of Hornsey et al. (1956) and converted to haem-Fe using the formula haem-Fe = hematin × atomic weight Fe/molecular weight hematin. Total Fe was determined by ICP-AES (Iris Intrepid II XSP, Thermo Electron corporation) following destruction by Bunsen burner and dry incineration at 550°C for 4h, followed by dissolving in 3 mL concentrated HNO_3, diluting to 10 mL HNO_3 and filtration. Total Fe was calculated based on a standard curve and expressed as mg/100g meat.

Oxidation products

TBARS concentrations in digests (-20°C) were measured by a modified method in accordance with Grotto et al. (2007). TBARS were formed from the reaction of MDA with 2-thiobarbituric acid in an acid environment. After extraction in 1-butanol, the absorbance of the colored

complex was measured colorimetrically at 532 nm. A standard curve with 1,1,3,3-tetramethoxypropane was used.

Levels of 4-HNE, pentanal, hexanal, heptanal, octanal and nonanal were analyzed in digests (-80°C) through HPLC (Agilent 1200 series, provided with a degasser, auto sampler, quaternary pump, column oven, fluorescence detector) using an adapted method of Holley et al. (1993). All solutions were purified with activated carbon and filtrated. For preparation of the cyclohexanedione (CHD) reagent, 10 g ammonium sulfate and 0.29 mL acetic acid were dissolved in 100 mL, adjusted to pH 5, and purified with activated carbon. After filtration, 0.25 g CHD, dissolved in 2 mL activated carbon purified MeOH, was added to the mixture. All glassware was cleaned with ethanol and H_2O and dried in a 100°C oven to remove contaminating aldehydes. One mL digest was mixed with 4 mL $CHCl_3$:MeOH (2:1, v/v) and 0.4 mL NaCl (0.9%). After 30s vortex, the mixture was centrifuged (5 min, 1100 g) and the $CHCl_3$ phase was collected. The remaining aqueous solution was mixed again with 2 mL $CHCl_3$:MeOH and the $CHCl_3$ phase was collected as described before. The combined $CHCl_3$ phases were dried under gentle N_2 stream. The dried residue was resolved in 0.1 mL MeOH, 0.4 mL H_2O and 1 mL CHD reagent. After 1 h of heating at 60°C in a warm water bath, 0.5 mL MeOH was added and following vortex, samples were filter sterilized (0.2 μm cellulose membrane filter) in dark HPLC vials. The injection volume was 80 μl and flow rate 1 mL/min. Separation was done on a Supelcosil LC-18 column (25 cm x 4.6 mm, 5 μm, cat. 58295 Supelco), using stepwise elution (50% tetrahydrofuran from 0-40 min). The derivatized aldehydes were detected by a fluorescence detector at an excitation wavelength of 380 nm and an emission wavelength of 446 nm. Aldehydes were quantified using a standard curve and expressed as pmol aldehyde / mL solution.

The measurement of PCC following their covalent reaction with 2,4-dinitrophenylhydrazine (DNPH) was done according to Ganhão et al. (2010). This reaction leads to the formation of a stable 2,4-dinitrophenyl hydrazone product. Total carbonyl content was quantified colorimetrically at 370 nm, using a molar absorption coefficient of 21.0/(mM·cm) and expressed as nmol DNPH / mg protein. Protein concentrations were measured at 280 nm after reaction with 2M HCl instead of DNPH, quantified using a standard curve with BSA and expressed as mg/mL solution. Both PCC and protein concentrations were reported separately.

O^6-Carboxymethylguanine (O^6-CMG)

The internal standard O^6-methyl-d3-guanine (O^6-d3-MeG) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). O^6-carboxymethyl-deoxyguanosine (O^6-CMdG) was a gift from S.A. Moore (Liverpool John Moores University, UK). The NOC-specific DNA adduct O^6-CMG was quantified using U-HPLC-MS/MS (Vanden Bussche et al., 2012). The chemical structure of this compound is shown in Figure 2.2. In brief, 182 µl of filter sterilized sample was incubated for 18 hours at 37°C with 100 µg calf thymus (CT)-DNA. After addition of the internal standard (50 µL, 20 ng/mL O^6-d3-MeG), the mixture was dissolved in 2 mL of 0.1M formic acid and hydrolyzed by heating (80°C for 30 min). The hydrolysate was cooled on ice and then applied to an Oasis HLB cartridge (SPE) (30 mg, 1 mL), which was conditioned with 2 mL of 100% MeOH and equilibrated with 2 mL deionized water. After loading the hydrolysate, a vacuum suction was applied on the SPE cartridge, followed by the elution step with 2 mL of 100% MeOH. The collected fraction was evaporated to dryness (90 min, 20°C) using a SpeedVac® Plus (Savant, Holbrook, NY, USA). Finally, the dried residue was dissolved in a total volume of 100 µl of mobile phase consisting of 95:5 0.05% aqueous acetic acid:H_2O. Chromatographic separation of the DNA-adducts was achieved by reversed phase

chromatography and gradient elution. Separation of the DNA-adducts was carried out on a Nucleodur C18 ISIS column (5 μm, 250 × 4 mm, Machery Nagel, Düren, Germany), kept at 30°C. Analysis was performed on a triple quadrupole mass analyzer (TSQ Vantage, Thermo Electron, San Jose, USA), fitted with a heated electrospray ionization (HESI-II) source operating in the positive ion mode. A standard curve of O^6-CMG was made by derivatization of O^6-CMdG with 0.1 M formic acid at 70°C for 1 hour (Moore et al., 2010). O^6-CMG was quantified using the standard curve and the area ratio of internal standard O^6-D3-MeG and expressed as ng O^6-CMG /mL digesta.

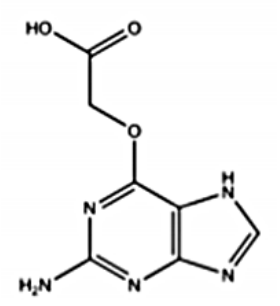

Figure 2.2: Chemical structure of O^6-carboxymethylguanine

Statistical analysis

For the meat characteristics, a linear model ANOVA procedure (SAS enterprise guide 5) was used with the fixed effects of haem-Fe, nitrite and haem-Fe × nitrite. Data on TBARS, 4-HNE, simple aldehydes, PCC and protein content were analyzed for the undigested meat, duodenum and colon step separately. For the undigested meat, the same linear model as for the meat characteristics was used, while for the duodenum and colon phase a mixed model ANOVA procedure was used with the fixed effects haem-Fe, nitrite and haem-Fe × nitrite and the random effect incubation run. Data on O^6-CMG were analyzed for each run (fecal inoculum) separately due to very high variation between the different applied inocula, using a linear model with the

fixed effects haem-Fe, nitrite and haem-Fe × nitrite. Tukey-adjusted *post hoc* tests were performed for all pairwise comparisons. P<0.05 was considered significant.

RESULTS

Meat characteristics

The characteristics of the used meats are shown in Table 1.2. Pork contained a marginally higher fat content compared to the other meats. The fatty acid profile showed no significant differences in SFA between the different meats, while minor differences were observed for total MUFAs and

Table 1.2: Composition of the meat model products used in the *in vitro* digestion experiment.

Nitrite-curing		Uncured			Nitrite-cured				P-values		
Haem-Fe		Chicken	Pork	Beef	Chicken	Pork	Beef	RMSE	H	NC	H×NC
	Unit										
Dry matter	%	30.1	31.9	30.6	31.3	31.9	30.0	0.45	**.027**	**.727**	.040
Protein	%	23.8	21.5	21.4	22.9	22.2	21.3	0.95	.055	.829	.526
Fat	%	4.7^{b}	5.6^{a}	5.3^{ab}	4.1^{b}	5.4^{a}	4.6^{b}	0.22	**< .001**	**.008**	.414
SFA	g/100g FAME	37.8	38.0	36.6	37.1	38.3	37.5	1.21	.466	.804	.686
MUFA	g/100g FAME	41.3^{ab}	42.2^{a}	39.6^{b}	40.3^{ab}	42.4^{a}	38.2^{c}	0.85	**.004**	.178	.474
PUFA	g/100g FAME	17.7^{a}	16.0^{c}	16.9^{b}	17.5^{a}	15.9^{b}	16.3^{b*}	0.20	**< .001**	**.021**	.301
ALA	g/100g FAME	0.80^{b}	0.69^{c}	0.90^{a}	0.79^{b}	0.65^{c}	0.89^{a}	0.015	**< .001**	.140	.504
LC *n*-3 PUFA	g/100g FAME	0.50^{b}	0.29^{c}	0.90^{a}	0.53^{b}	0.27^{c}	0.91^{a}	0.037	**< .001**	.919	.568
LA	g/100g FAME	14.1^{a}	12.9^{b}	12.7^{b}	13.8^{a}	12.8^{b}	12.1^{c*}	0.210	**< .001**	**.023**	.252
LC *n*-6 PUFA	g/100g FAME	1.43^{b}	1.36^{b}	1.75^{a}	1.47^{b}	1.38^{b}	1.76^{a}	0.055	**< .001**	.491	.917
Residual nitrite	mg/100g	-	-	-	6.00^{a}	3.64^{b}	1.85^{c}	0.058	**< .001**	-	-
Total Fe	mg/100g	0.43^{b}	0.55^{b}	1.38^{a}	0.41^{b}	0.49^{b}	1.44^{a}	0.079	**< .001**	.894	.549
Haem-Fe	mg/100g	0.14^{b}	0.25^{b}	1.33^{a}	0.15^{b}	0.31^{b}	1.34^{a}	0.044	**< .001**	.433	.844

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; ALA = α-linolenic acid (C18:3, *n*-3); LC *n*-3 PUFA = long chain omega-3 polyunsaturated fatty acids (C20:5, *n*-3; C22:5, *n*-3; C22-6, *n*-3); LA = linoleic acid (C18:2, *n*-6); LC *n*-6 PUFA = Long chain omega-6 polyunsaturated fatty acids (C20:4, *n*-6; C22:4, *n*-6; C22:5, *n*-6), FAME = fatty acid methyl esters; RMSE = root mean square error; H = Haem-Fe content; NC = Nitrite-curing; a,b,c = means for different haem-Fe content (within curing treatment) with different superscripts are significantly different (P < 0.05); * = significantly different from uncured equivalent (P < 0.05)

PUFAs. The beef sample contained significantly more ALA, LC n-3 PUFA and LC n-6 PUFA and less LA compared to the other meats, whereas the chicken sample contained significantly more ALA and LC *n*-3 PUFA compared to pork. A clearly lower amount of residual nitrite in cured meat samples was found, compared to the added amount (120 mg/kg meat). The lowest amount of residual nitrite was found in beef, followed by pork and chicken. Beef had significantly higher haem-Fe and total Fe compared to chicken and pork.

Lipid oxidation

Some illustrative HPLC chromatograms are shown in Figure 2.2 and 2.3.

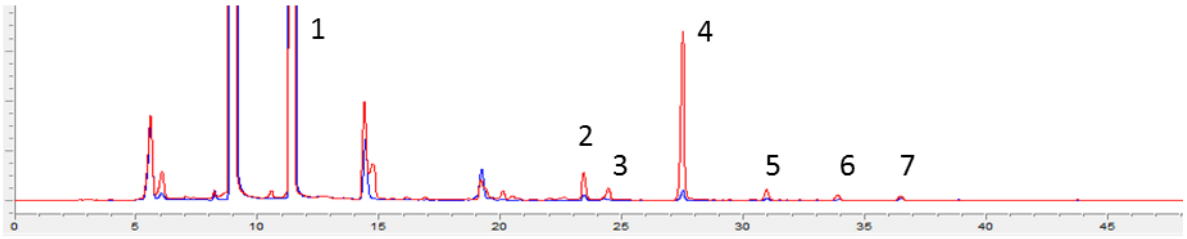

Figure 2.2: HPLC chromatograms of an uncured beef digest (red) *vs.* nitrite-cured beef digest (blue). 1. Malondialdehyde + acetaldehyde; 2. Pentanal; 3. 4-hydroxy-nonenal; 4. Hexanal; 5. Heptanal; 6. Octanal; 7. Nonanal.

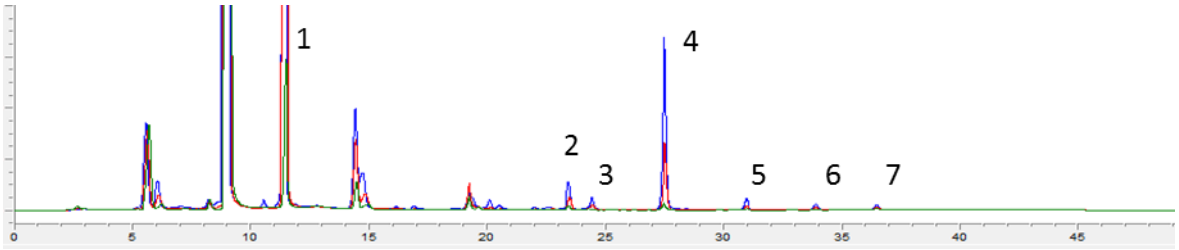

Figure 2.3: HPLC chromatograms of an uncured beef digest (blue) *vs.* uncured pork digest (red) *vs.* uncured chicken digest (green). 1. Malondialdehyde + acetaldehyde; 2. Pentanal; 3. 4-hydroxy-nonenal; 4. Hexanal; 5. Heptanal; 6. Octanal; 7. Nonanal.

Table 1.3 shows the concentrations of LOP before the digestion and after duodenal and colonic digestion of the meats. Uncured beef showed significantly higher amounts of all LOP before

digestion, except for nonanal, compared to pork, followed by chicken. Concentrations of LOP were significantly lower when meats were nitrite-cured to even undetected for 4-HNE and heptanal. Compared to uncured chicken, nitrite-cured chicken had significantly lower TBARS and hexanal concentrations, whereas 4-HNE and heptanal concentrations were hardly detectable. An increase in all LOP was observed in the duodenum step, however this was less distinctive for 4-HNE. Also after duodenal digestion, uncured beef resulted in significantly higher concentrations of

Table 1.3: Lipid oxidation in uncured and nitrite-cured chicken, pork and beef before and after *in vitro* digestion.

Nitrite-curing		Uncured			Nitrite-cured				P-Values		
Haem-Fe		Chicken	Pork	Beef	Chicken	Pork	Beef	RMSE	H	NC	H×NC
	Step										
TBARS	BD	5.7^{b}	5.4^{b}	15.1^{a}	2.8^{b*}	2.0^{b*}	7.4^{a*}	0.41	**< .001**	**< .001**	**< .001**
(nmol/mL)	D	9.8^{c}	14.8^{b}	28.1^{a}	8.7^{b}	9.2^{b*}	11.0^{a*}	0.96	**< .001**	**< .001**	**< .001**
	C	13.9^{c}	17.7^{b}	26.3^{a}	12.8^{b}	13.0^{b*}	15.3^{a*}	1.21	**< .001**	**< .001**	**< .001**
4-HNE	BD	48.5^{c}	221.2^{b}	428.6^{a}	8.8	*nd*	*nd*	48.08	**.004**		
(pmol/mL)	D	20.4^{c}	211.6^{b}	521.2^{a}	12.2	19.4*	19.8*	69.57	**< .001**	**< .001**	**< .001**
	C	16.4^{b}	20.2^{ab}	23.6^{a}	15.8	17.1	13.3*	2.90	**.088**	**< .001**	**< .001**
Pentanal	BD	65^{c}	172^{b}	331^{a}	84^{a}	32^{b*}	37^{b*}	10.8	**< .001**	**< .001**	**< .001**
(pmol/mL)	D	130^{c}	314^{b}	697^{a}	103	124*	107*	66.2	**< .001**	**< .001**	**< .001**
	C	108	126	125	101	106	87*	14.03	.098	**< .001**	**< .037**
Hexanal	BD	287^{c}	1162^{b}	1391^{a}	107*	77*	77*	32.4	**< .001**	**< .001**	**< .001**
(pmol/mL)	D	299^{c}	1017^{b}	2652^{a}	214	212*	232*	178.5	**< .001**	**< .001**	**< .001**
	C	178^{b}	209^{ab}	241^{a}	176^{ab}	211^{a}	138^{b*}	25.0	**.011**	**< .001**	**< .001**
Heptanal	BD	3.0^{c}	18.4^{b}	56.0^{a}	1.9	*nd*	*nd*	2.24	**< .001**		
(pmol/mL)	D	23.2^{bc}	65.0^{b}	147.8^{a}	20.8	16.1	23.9*	34.22	**< .001**	**< .001**	**< .001**
	C	84.2^{a}	67.5^{a*}	38.9^{b}	99.1^{a}	132.0^{a}	69.5^{b}	25.35	**< .001**	**< .001**	.065
Nonanal	BD	10.1	14.9	16.9	12.7	10.3	6.8	3.03	.866	.060	.067
(pmol/mL)	D	21.6^{c}	28.9^{b}	52.2^{a}	16.0^{b}	16.9^{b*}	24.3^{a*}	3.12	**< .001**	**< .001**	**< .001**
	C	8.5	7.8	14.1	10.0	8.8	13.3	3.75	**.005**	.682	.754

TBARS = thiobarbituric acid reactive substances; 4-HNE = 4-hydroxy-2-nonenal; BD = before digestion (total n=12); D = duodenal stage (total n=36); C = colonic stage (total n=36); RMSE = root mean square error; H = Haem-Fe content; NC = Nitrite-curing; a,b,c = means for different haem-Fe contents (within curing treatment) with different superscripts are significantly different ($P < 0.05$); * = significantly different from uncured equivalent ($P < 0.05$); *nd* = not detected.

LOP compared to pork, followed by chicken. Hexanal concentrations in duodenal digested uncured beef increased 2-fold during digestion while the hexanal concentrations in the uncured chicken and pork remained similar. Nonanal increased 3-fold during digestion of uncured beef, while only 2-fold in uncured chicken and pork. After duodenal digestion of nitrite-cured meats, 4-HNE and heptanal were detected, but in accordance to the other LOP in much lower concentrations compared to their uncured equivalents. Except for TBARS, the concentration of LOP was clearly lower after 72 h of colonic digestion compared to the duodenum step or before digestion. Similar significant effects of nitrite-curing and haem-Fe content on most LOP concentrations were observed in the colon digestive samples. In contrast to other LOP in the colonic digestive fluids, heptanal was significantly lower in beef compared to chicken and pork and significantly higher in nitrite-cured meats compared to the uncured equivalents. Nitrite-curing had no significant effect on nonanal in the colonic digestion samples. Octanal was not detected or close to no detection and hence was not included in the statistical analysis.

Protein oxidation

Undigested nitrite-cured meats had significantly lower PCC concentrations than its uncured equivalents (Table 1.4). Higher PCC concentrations were observed after duodenal and colonic digestion with significantly higher PCC levels in uncured beef digests compared to chicken and pork after duodenum digestion and compared to chicken after colon digestion. Nitrite-curing of the meat also resulted in lower protein oxidation in simulated duodenum and in marginally lower values in the colonic step.

A clear decrease in protein levels was observed starting from the non-digested meat to the duodenal and colonic digestive fluids (Table 4). No significant differences were observed between different meat samples before digestion. Cured meats had significantly lower protein levels in

duodenum and colon compared to uncured equivalents. A marginal effect of haem-Fe was also observed in the colonic phase.

Table 1.4: Protein oxidation in uncured and nitrite-cured chicken, pork and beef before and after *in vitro* digestion.

Nitrite-curing		Uncured			Nitrite-cured				P-Values		
Haem-Fe		Chicken	Pork	Beef	Chicken	Pork	Beef	RMSE	H	NC	H×NC
	Step										
PCC	BD	1.57	1.85	2.17	0.65*	0.76*	0.88*	0.279	.082	**< .001**	.562
(nmol DNPH/	D	2.47[b]	2.81[b]	4.87[a]	1.78	1.79*	1.97*	0.445	**< .001**	**< .001**	**< .001**
mg protein)	C	3.11[b]	3.54[ab]	4.19[a]	2.93	3.10	3.71	0.517	**< .001**	**.043**	.742
Protein	BD	6.44	6.44	6.05	6.40	6.09	6.20	0.580	.851	.865	.875
(mg/mL)	D	2.22	2.28	2.41	2.24	1.98	2.05	0.207	.497	**.017**	.174
	C	1.54	1.36	1.38	1.32*	1.26	1.25	0.109	**.020**	**< .001**	.332

PCC = protein carbonyl compounds; BD = before digestion (total n=12); D = duodenal stage (total n=36); C = colonic stage (total n=36); RMSE = root mean square error; H = haem-Fe content; NC = Nitrite-curing; a,b,c = means for different haem-Fe contents (within curing treatment) with different superscripts are significantly different ($P < 0.05$); * = significantly different from uncured equivalent ($P < 0.05$)

O^6-Carboxymethylguanine

No O^6-CMG was detected in meats before digestion or at the end of the duodenal phase (Table 1.5). In contrast to the other parameters, detection of O^6-CMG after 72 h of colonic digestion was highly dependent on the individual bacterial inoculum. Inoculum originating from the first test subject did not allow detection of O^6-CMG in any sample. When fermented using inoculum originating from the second and third test person, O^6-CMG levels were low or very high, respectively. After 72 h of fermentation, a significant effect of haem-Fe concentration was observed. When fermented by inoculum 2, uncured beef had significantly more O^6-CMG than chicken while no difference was seen in nitrite-cured meats. Fermentation by the third inoculum resulted in higher O^6-CMG formation in both uncured and nitrite-cured beef compared to chicken and pork. No significant effect of nitrite-curing was found.

Table 1.5: NOC-induced DNA damage by uncured and nitrite-cured chicken, pork and beef before and after *in vitro* digestion.

Nitrite-curing		Uncured			Nitrite-cured				P-Values		
Haem-Fe		Chicken	Pork	Beef	Chicken	Pork	Beef	RMSE	H	NC	H×NC
	Step										
O⁶-CMG	BD	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*				
(ng/mL)	D	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*				
	C1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*				
	C2	10.8^{b}	13.3^{ab}	14.4^{a}	11.9	11.9	11.9	0.89	.071	.121	.066
	C3	539^{b}	504^{b}	762^{a}	558^{b}	620^{b}	760^{a}	45.4	**.001**	.143	.225

O⁶-CMG = O⁶-carboxymethylguanine; BD = before digestion (total n=12); D = duodenal stage (total n=36); C1, C2, C3 = colonic stage incubated with fecal inoculum 1, 2 and 3 (total n=12 per fecal inoculum); RMSE = root mean square error; H = Haem-Fe content; NC = Nitrite-curing; a,b,c = means for different haem-Fe contents (within curing treatment) with different superscripts are significantly different ($P < 0.05$); *nd* = not detected

DISCUSSION

The objective of the present study was to test the involvement of haem-Fe and nitrite-curing on the formation of toxic compounds during the digestion of meat. In uncured meats, the promoting effects of haem-Fe on oxidation and NOC-induced DNA damage during digestion were distinct. Our data confirm the importance of haem-Fe as catalyst of endogenous oxidation and nitrosation reactions, and may hence contributeto the association between red meat consumption and CRC. Curing with nitrite drastically inhibited the formation of the major toxic oxidation products MDA, 4-HNE, the minor toxic simple aldehydes and PCC with no stimulating effect on the formation of NOC-derivative DNA adducts. Consequently, nitrite-curing of meat was not perceived as a risk factor in the presented study.

In accordance to our results, Hur et al. (2009) already demonstrated a rise in TBARS during *in vitro* digestion of beef patties until duodenal digestion. Increased oxidation during digestion of meat under simulated duodenal conditions was confirmed in our study using more specific markers. Changes in 4-HNE concentrations before and after duodenal digestion were less

distinct compared to other LOP. However, measurable levels of 4-HNE were noted in nitrite-cured meat samples under simulated duodenal and colonic conditions, while it was not detected before digestion, indicating the formation of 4-HNE during digestion. Gorelik et al. (2008) demonstrated absorption and accumulation of MDA in human plasma and urine after consuming turkey cutlets. Increased plasma 4-hydroxy-2-hexenal (4-HHE) in mice after consuming oxidized *n*-3 fatty acids, was associated with oxidative stress and inflammation in the upper intestine (Awada et al., 2012). Through systemic circulation, LOP can reach sensitive tissues where MDA and 4-HNE can form adducts with DNA, increasing cancer risk (Nair et al., 2007). In this way, higher plasma and tissue MDA in rats fed with beef compared to chicken, were associated with higher colonic DNA strand breaks (Toden et al., 2010). In our experiment, the clearly lower concentrations of LOP after colonic digestion, with an exception for MDA, could be attributed to degradation into volatile compounds, and adduct formation with proteins or bacterial DNA. Oxidation of aldehydes by previously demonstrated aldehyde dehydrogenase activity of colonic bacteria (Nosova et al., 1996) could also explain the observed decrease throughout colonic digestion. It should be noted that the fecal inocula likely also contained aldehydes. The net disappearance of the aldehydes during colonic digestion was thus probably underestimated.

The antioxidative effect of nitrite was clearly demonstrated in meats before and after digestion. Whereas high concentrations of the major cytotoxic 4-HNE were found in uncured beef, undetected to very low traces were observed for nitrite-cured beef. Nitrite-curing of meat samples was performed by mixing with 0.6% nitrite salt. In contrast, adding salt without nitrite to meat increased TBARS and peroxides in meat (Gheisari et al., 2010). Adding nitrite (1 g/l) in drinking water significantly reduced lipid oxidation in the colon of rats on a haem-diet (Chenni et al., 2013). These results should be interpreted with care since a dual role of ·NO on oxidative

stress has been described. Since a 1:1 ratio of ·NO to ROS enhances lipid peroxidation while an excess of ·NO inhibits oxidation (Darley-Usmar et al., 1995), consumption of oxidized nitrite-cured meat could result in a reversed effect. In contrast to other aldehydes in the colonic digests, heptanal was surprisingly lower in beef compared to chicken and pork and higher in nitrite-cured meats compared to their uncured equivalents. This unexpected result should be clarified in future experiments.

Because LOP arise from PUFAs, we aimed to exclude a possible confounding effect of different fatty acid profiles by adding 4% subcutaneous pork fat to the lean chicken, pork and beef meats (approximately 1% intramuscular fat) of the present study. Despite this, there were some minor differences in the PUFA subgroups. Total PUFAs was very similar across the different samples. 4-HNE and hexanal arise specifically from *n*-6 PUFA (Esterbauer et al., 1991). The small differences in *n*-6 PUFA content among the different meat samples in the present study are not expected to explain the large differences in 4-HNE and hexanal formation. LA, which belongs to the *n*-6 PUFA subgroup, was even higher in the chicken meat, but was associated with the lowest 4-HNE and hexanal formation.

Measurement of PCC as marker for protein oxidation compares well with our observations on lipid oxidation. Higher haem-Fe concentrations in meats induced more protein oxidation while nitrite-curing prevented oxidation before and during *in vitro* digestion. It was previously demonstrated that CRC patients had higher plasma PCC levels than healthy controls (Yeh et al., 2010). The formation of PCC affects the nature and function of the protein but the importance of dietary PCC to human health is still not completely clear. As protein oxidation is believed to be a free radical chain reaction (Lund et al., 2011), it could be hypothesized that oxidized dietary proteins catalyze the oxidation of cellular proteins and hence induce cell damage.

The significantly lower PCC and protein levels in nitrite-cured meats compared to uncured meats during simulation of duodenum and colon digestion agree with Santé-Lhoutellier et al. (2008) who found a highly significant negative correlation between PCC concentrations and proteolytic susceptibility to pepsin. 4-HNE can also react with proteins through Michael-addition and Schiff base formation (Petersen and Doorn, 2004), which are known to play a role in protein aggregation. Previously, it was shown that a higher amount of protein reaching the colon fermentation, resulted in a higher formation of potentially toxic protein fermentation products such as ammonia, phenol, *p*-cresol and indol. However, higher formation of these products was not associated with enhanced colon cancer promotion in rats (Corpet et al., 1995).

The increased O^6-CMG concentrations in colon samples when meats contained higher haem-Fe contents were in accordance to Bingham et al. (2002) who showed a dose-responsive increase in ATNC with consumption of red meat, while consumption of large amounts of white meat did not influence fecal ATNC. Cross et al. (2003) showed that haem-Fe was responsible for endogenous intestinal *N*-nitrosation arising from red meat. Elevated O^6-CMG were also observed in colonic exfoliated cells of humans on a high red meat diet (Lewin et al., 2006). These observations are in agreement with extensive epidemiologic studies that showed a significantly increased CRC risk with high red meat consumption (Larsson & Wolk, 2006; Chan et al., 2011). Ijssennagger et al. (2012) provided evidence for selective susceptibility of Gram-positive bacteria to haem cytotoxic fecal water, which was not observed for Gram-negative bacteria. Furthermore, haem-Fe increased colonic *Enterobacteria* and *Bacteroidetes* spp. and decreased *Firmicutes* spp., among which *Lactobacilli* spp. (Ijssennagger et al., 2012; Schepens et al., 2011). Possibly, a bacterial shift induced by haem-Fe toxicity or haem-induced oxidative stress could alter the bacterial metabolic capacity and contribute to higher O^6-CMG formation in beef digesta. The pronounced

differences in the detection of the NOC-derivative DNA adduct O^6-CMG among the individual fecal inocula are most likely the result of variation in the gut bacterial composition of the fecal donor or varying concentrations of compounds in the fecal inoculum that are involved in formation of the DNA adduct O^6-CMG. More research is needed to clarify how and which bacterial species or compounds are responsible for the formation of O^6-CMG.

A higher formation of O^6-CMG when meats were nitrite-cured was not observed and hence, our results do not support an earlier suggestion about the importance of nitrite-curing on NOC-formation (Corpet, 2011). Rats on a high haem-Fe diet displayed threefold higher fecal ATNC when drinking water contained sodium nitrate and nitrite (0.17 g/l $NaNO_2$ and 0.23 g/l $NaNO_3$) (Chenni et al., 2013). However, since the increase in ATNC was mainly attributed to an increase of the non-genotoxic nitrosyl-Fe, the authors concluded this was probably not associated with an increased CRC risk. Nitrite-cured meats also contain far less residual nitrite than many vegetables, which are not associated with increased risk in epidemiologic studies. Furthermore, nitrite is formed endogenously and recycled with the saliva. Santarelli et al. (2010) showed clearly higher fecal ATNC in 1,2-dimethylhydrazin injected rats on a diet of dark, nitrite-cured, cooked and oxidized meat. In contrast, no increase was observed in rats on a diet of dark, nitrite-cured, cooked and anaerobic stored meat. Meats used in our experiment were stored anaerobically at -20°C to prevent oxidation during storage. Our results did not show an increased O^6-CMG formation when nitrite-cured meats were stored properly.

In conclusion, our study presents a valuable *in vitro* tool to study oxidation and nitrosation processes during digestion of meat. Our results confirm an earlier described catalyzing effect of haem-Fe in meat on oxidative stress and demonstrate NOC-induced DNA damage during

digestion. Nitrite-curing of meat resulted in a lower formation of toxic oxidation products while no effect was observed on the formation of the NOC-derivative DNA adduct O^6-CMG.

Chapter II

Fat content and nitrite-curing influence the formation of oxidation products and NOC-derivative DNA adducts during *in vitro* digestion of meat

Adapted from:

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ABSTRACT

The effects of fat content and nitrite-curing of pork were investigated on the formation of cytotoxic and genotoxic lipid oxidation products (MDA, 4-HNE, volatile simple aldehydes), protein oxidation products (PCC) and NOC-derivative DNA adducts (O^6-CMG) during *in vitro* digestion. The formation of these products during digestion is suggested to be responsible for the association between red (processed) meat consumption and CRC risk. Digestion of uncured pork to which fat was added (total fat content 5 or 20%), resulted in significantly higher lipid and protein oxidation in the mimicked duodenal and colonic fluids, compared to digestion of pork without added fat (1% fat). A higher fat content also significantly favored the formation of O^6-CMG in the colon. Nitrite-curing of meat resulted in significantly lower lipid and protein oxidation before and after digestion, while an inconsistent effect on the formation of O^6-CMG was observed. The presented results demonstrate that haem-Fe is not solely responsible for oxidation and nitrosation reactions throughout an *in vitro* digestion approach, but its effect is promoted by a higher fat content in meat.

INTRODUCTION

Several meta-analyses have reported a significant epidemiological association between CRC and high intake of red (processed) meat (Larsson & Wolk, 2006; Chan et al., 2011). However, the biochemical mechanisms underlying this association have not been completely elucidated yet. To date, the formation of cyto- and genotoxic LOP and genotoxic NOCs during digestion are considered the most plausible factors contributing to the increased risk of developing CRC when consuming large amounts of red and in particular processed meat (Corpet, 2011). Meat contains proteins, unsaturated lipids, haem-Fe and in case of processed meat also nitrite, which are all

compounds involved in oxidation and nitrosation processes. Haem-Fe in meat has been shown to be the main promoter of the peroxidation and nitrosation pathway (Corpet, 2011). However, when rats were fed a diet rich in haem-Fe, the effect of haem on colonic epithelial proliferation and cytolytic activity of fecal water was lower in a low fat diet (4.2%), compared to diets containing more fat (11.5 and 20.3%) (Sesink et al., 2000). A lean beef diet (5.0% fat) high in haem-Fe did not promote colon carcinogenesis in cancer-induced rats (Lai et al., 1997). Hence, we hypothesized that the fat content in meat might modulate the effect of haem on peroxidation and nitrosation.

Nitrite salt, a widely used curing agent in meat products, has been suggested to increase NOC formation in meat products. Several studies have reported on the influence of nitrite-cured meats on the promotion of CRC. Rats consuming nitrite-cured hot-dogs (4.7% fat) had 3.7 to 5.0 fold higher NOC levels in feces compared to a control group, while consumption of beef (3.6% fat) resulted in a 2.0 to 2.9 fold increase (Mirvish et al., 2002). A nitrite-cured ham diet increased cytotoxicity, lipid peroxidation and amount of 'mucin depleted foci' (MDF) in the colon of rats, compared to a control group (Pierre et al., 2010). However, fresh meat and processed meats differ in more properties than only nitrite-curing. Variation in haem-Fe content, fat content, and different processing procedures such as mincing and heating do not allow to draw conclusions on the isolated effect of nitrite-curing in previously mentioned studies. In a controlled study, Santarelli et al. (2010) showed that consumption of nitrite-cured meat and oxidized meat increased ATNC and MDF in the colon of rats compared to consumption of similar non-cured and non-oxidized meat. Moreover, a human intervention study showed increased fecal ATNC when a high proportion of red meat (21.4% fat) or nitrite-cured processed meat (24.0% fat) was consumed, compared to a vegetarian diet, but no significant difference was seen between the red meat and processed meat diets (Joosen et al., 2009). Therefore, we aimed at elucidating the likely modulating effects of fat content and nitrite-curing of meat on lipid and protein oxidation markers, and the formation of

NOC-derivative DNA adducts during digestion. For this purpose, we used an *in vitro* digestion protocol, as described in Chapter I.

MATERIALS AND METHODS

Experimental set-up

Uncured and nitrite-cured heated pork samples differing in fat content (targeted at 1, 5 or 20% fat) were compared before and after mimicked duodenal and colonic digestion. Each incubation run included all treatments in quadruplicate from which 2 duplicates underwent *in vitro* digestion until the duodenum and the remaining 2 until the colon. Each incubation run was performed three times with fecal inoculum originating from three different persons. In total, 2 meat samples before digestion, 6 duodenal digests and 6 colonic digests were obtained for each of the 6 prepared meat products.

Preparation of the meat samples

Commercially available lean meat samples from the *m. Longissimus dorsi* of pig were purchased in a local supermarket. The loin was manually chopped into cubes of approximately 1-2 cm^3. Subcutaneous pork fat from one batch was added to the chopped meat to obtain a targeted total fat content of 1 (no fat added), 5 and 20%. Subsequently, meat samples were first minced in a grinder (Omega T-12) equipped with a 10 mm plate, followed by grinding through a 3.5 mm plate. After grinding, nitrite-curing was applied by adding 20 g 0.6% nitrite salt/kg meat, corresponding to an added concentration of 120 mg nitrite/kg meat. All meat samples were heated in a warm water bath for 15 min after the core temperature had reached 65°C. After

manufacturing, all meat samples were homogenized in three 5 s bursts using a food processor (Moulinex DP700), vacuum packed and stored at -20°C until the start of the incubation.

Digestive simulations

See Chapter I (p. 21).

Preparation of human fecal inoculum

See Chapter I (p. 23).

Chemical composition of the meat samples

See Chapter I (p. 25).

Oxidation products

See Chapter I (p. 25).

O^6-Carboxymethylguanine

See Chapter I (p. 27).

Short chain fatty acids and NH_3

Five ml of 10% formic acid containing internal standard (1 mg 2-ethyl butyric acid) was added to 1 ml digest. After 15 min centrifugation at 4°C and 22.000 *g*, supernatant was filtered and an aliquot was transferred into a 1.5 ml glass vial. Samples were stored at 4°C until analysis using gas chromatography on a Shimadzu 2010 (Shimadzu Corporation, 's-Hertogenbosch, The Netherlands) equipped with a Nukol column (30 m × 0.25 mm × 0.25 µm, Supelco) with a flame ionization detector. Briefly, 0.5 µl of sample was injected with the carrier gas N_2, the injector temperature was 250°C and the inlet pressure 52.7 kPa. The temperature program was 90 °C at

the start of the injection, increasing 20°C/min until 160°C (kept for 8.5 min), increasing 10°C/min until 170°C (kept for 2 min) (Castro-Montoya et al., 2012). The detection temperature was 250°C. Ammonia concentrations were measured by a colorimetrical method described by Chaney and Marbach (1962).

Statistical analysis

Data were analyzed using SAS enterprise guide 5. For the data on the meat samples prior to digestion, a linear model with the fixed effects of fat content, nitrite-curing and fat content × nitrite was used. For the duodenal and colonic digestion samples, a mixed model was used with the same fixed effects and the random effect of incubation run. Data on O^6-CMG were analyzed per incubation run separately due to very high variation between runs related to the use of different human fecal inocula. Tukey-adjusted *post hoc* tests were performed for all pairwise comparisons. $P<0.05$ was considered significant.

RESULTS

Meat characteristics

The characteristics of the prepared meats prior to digestion are shown in Table 2.1. Dry matter content and fatty acid concentrations increased, whereas protein content decreased with increasing fat content. There was an unexpected effect of nitrite and fat content × nitrite interaction for the fat content due to a lower than targeted fat content of the 20% fat uncured samples. Addition of subcutaneous pork fat to the lean meat resulted in a significantly lower proportion of LC *n*-3 and *n*-6 PUFA and higher ALA, LA and SFA proportions in the fatty acid profile of the prepared meat

samples. Residual nitrite levels in the cured meat samples amounted to approximately 41%, 30% and 17% of the added amount (120 mg/kg meat), hence there was a significant effect of fat content on the residual nitrite level. Even though originating from the same pork batch, nitrite-cured meats contained significantly less Fe than the corresponding uncured meats. However, no effect was observed on haem-Fe. The pH was slightly lower in the nitrite-cured meats while no effect of fat content was observed.

Table 2.1: Composition of the pork model products used in the *in vitro* digestion

Nitrite-curing		**Uncured**			**Nitrite-cured**				**P-values**		
Fat content (%)		**1**	**5**	**20**	**1**	**5**	**20**	**RMSE**	**F**	**NC**	**F×NC**
Item	**Unit**										
Dry matter	%	29.1^{c}	31.9^{b}	39.0^{a}	29.3^{c}	31.1^{b}	43.6^{a*}	0.41	**< .001**	**.002**	**< .001**
Protein	%	24.5^{a}	21.5	17.8^{b}	22.2	22.2	18.4	1.08	**.001**	.597	.171
Fat	%	1.6^{c}	5.6^{b}	17.7^{a}	1.7^{c}	5.4^{b}	20.6^{a*}	0.38	**< .001**	**.006**	**< .001**
SFA	g/100g FAME	35.0^{c}	38.0^{b}	39.6^{a}	38.3^{b*}	38.3^{b}	39.6^{a}	0.22	**.026**	**< .001**	**< .001**
MUFA	g/100g FAME	41.3	42.2	43.1	41.5	42.2	42.9	0.91	.178	.958	.942
PUFA	g/100g FAME	15.4	16.0	16.4	15.4	15.9	16.1	0.23	.101	**.042**	.348
ALA	g/100g FAME	0.32^{b}	0.68^{a}	0.79^{a}	0.31^{b}	0.69^{a}	0.75^{a}	0.02	**< .001**	.209	.868
LC *n*-3 PUFA	g/100g FAME	0.86^{a}	0.29^{b}	0.22^{b}	0.81^{a}	0.27^{b}	0.17^{b}	0.02	**.002**	.058	.793
LA	g/100g FAME	10.7^{b}	12.9^{a}	13.9^{a}	11.3	12.8	13.7	0.52	**< .001**	.757	.678
LC *n*-6 PUFA	g/100g FAME	3.84^{c}	1.35^{b}	0.69^{a}	3.19^{c*}	1.38^{b}	0.69^{a}	0.07	**< .001**	**.005**	**.003**
Residual nitrite	mg/100g	-	-	-	4.87^{c}	3.64^{b}	1.98^{a}	0.078	**< .001**		
Total Fe	mg/100g	0.54	0.55	0.61	0.46	0.49	0.49	0.033	**.**238	**.004**	**.**495
Haem-Fe	mg/100g	0.26	0.25	0.26	0.29	0.31	0.26	0.118	.627	.291	.672
pH	-	5.6	5.6	5.6	5.5	5.5	5.5				

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; ALA = α-linolenic acid (C18:3, *n*-3); LC *n*-3 PUFA = long chain omega-3 polyunsaturated fatty acids (C20:5, *n*-3; C22:5, *n*-3; C22-6, *n*-3); LA = linoleic acid (C18:2, *n*-6); LC *n*-6 PUFA = Long chain omega-6 polyunsaturated fatty acids (C20:4, *n*-6; C22:4, *n*-6; C22:5, *n*-6); FAME = fatty acid methyl esters; RMSE = root mean square error; a,b,c = means for different fat content (within curing treatment) with different superscripts are significantly different ($P < 0.05$); * = significantly different from uncured equivalent ($P < 0.05$).

Lipid oxidation

In the meat samples prior to digestion, TBARS, pentanal and hexanal were significantly affected by fat content and nitrite-curing, the interaction term appeared also significant (Table 2.2). TBARS was by far the most abundant aldehyde. Octanal was not detected at all, whereas 4-HNE and heptanal were detected in uncured but not in nitrite-cured meats. The concentration of nonanal

was not affected by fat content, but nitrite-curing had a significant effect. TBARS and volatile aldehyde concentrations were at least 2-fold up to 20-fold lower in the nitrite-cured versus the uncured meat samples. Aldehyde concentrations generally increased with increasing fat content in the uncured samples, but no clear effect of fat content on the aldehyde concentrations in the nitrite-cured samples was observed.

Table 2.2: Lipid aldehyde concentrations in uncured and nitrite-cured pork containing different amounts of fat (1, 5, 20%) before and after *in vitro* digestion

Nitrite-curing		Uncured			Nitrite-cured				P-Values		
Fat content (%)		1	5	20	1	5	20	RMSE	F	NC	F×NC
	Phase										
TBARS	BD	4.0^{b}	5.4^{a}	5.3^{a}	2.0*	2.0*	1.9*	0.19	**< .001**	**< .001**	**< .001**
(nmol/ml)	D	9.1^{b}	13.3^{a}	12.6^{a}	8.2*	8.4*	8.0*	0.51	**< .001**	**< .001**	**< .001**
	C	15.5^{b}	18.0^{a}	16.4^{b}	14.7	14.3*	13.4*	0.85	**< .001**	**< .001**	**< .001**
4-HNE	BD	58^{b}	221^{a}	290^{a}	*nd*	*nd*	*nd*	38.1	**.019**		
(pmol/ml)	D	3.2^{c}	143^{b}	192^{a}	*nd*	11.5^{b*}	77.2^{a*}	31.6	**< .001**	**< .001**	**< .001**
	C	13.2^{b}	28.2^{a}	35.5^{a}	14.1	19.5	23.3	10.6	**.005**	.079	.335
Pentanal	BD	87^{b}	172^{a}	192^{a}	38	32*	45*	15.1	**.005**	**< .001**	**.007**
(pmol/ml)	D	65^{b}	220^{a}	211^{a}	56^{b}	91^{ab*}	124^{a*}	30.7	**< .001**	**< .001**	**< .001**
	C	73^{b}	115^{ab}	123^{a}	75^{b}	89^{ab}	121^{a}	25.3	**< .001**	.291	.352
Hexanal	BD	286^{b}	1162^{a}	1425^{a}	75	77*	68*	155.5	**.005**	**< .001**	**.005**
(pmol/ml)	D	158^{b}	581^{a}	604^{a}	106	154*	219*	126.3	**< .001**	**< .001**	**.002**
	C	121^{b}	229^{a}	252^{a}	116	156	200	61.4	**< .001**	**.044**	.384
Heptanal	BD	18.4	18.4	24.4	*nd*	*nd*	*nd*	2.91	.202		
(pmol/ml)	D	14.5^{b}	37.7^{a}	27.1^{x}	9.7	13.4*	11.3*	7.86	**< .001**	**< .001**	**.021**
	C	38.2	90.6	90.5	28.5^{b}	74.7^{ab}	105.8^{a}	35.70	**< .001**	.779	.537
Nonanal	BD	20.2	14.9	18.6	6.5*	10.2	5.8*	2.92	.838	**< .001**	.128
(pmol/ml)	D	18.6	18.4	12.9	18.6^{a}	13.1^{ab}	10.4^{b}	3.42	**< .001**	**.029**	.179
	C	11.7^{b}	20.2^{a}	24.3^{a}	12.4	15.4	19.4	4.70	**< .001**	.064	.261

Thiobarbituric acid reactive substances (TBARS) were measured colorimetrically and expressed as nmol/ml digest. 4-hydroxy-nonenal (4-HNE) and the simple aldehydes were measured through HPLC-fluorescence and expressed as pmol/ml. Aldehydes in meat before digestion (BD) (total n=12) were analyzed using a linear model with the fixed effects of fat content (F), nitrite-curing (NC) and their interaction term (F×NC). Aldehydes in duodenal (D) digests (total n=36) and colonic (C) digests (total n=36) were analyzed using a mixed model with the fixed effects of F, NC and F×NC and the random effect of incubation run. RMSE= root mean square error; a,b,c = means for different fat content (within curing treatment) with different superscripts are significantly different ($P < 0.05$); * = significantly different from uncured equivalent ($P < 0.05$); *nd* = not detected

After digestion, TBARS was by far the most abundant aldehyde, followed by hexanal. The effects of fat content, nitrite-curing and their interaction term were significant for most aldehydes. Nitrite-curing had no significant effect on pentanal and heptanal concentrations after colonic digestion. Higher concentrations of TBARS were measured in both uncured and nitrite-cured meats compared to undigested meat. In contrast, concentrations of 4-HNE and volatile aldehydes were lower or similar after digestion of uncured meats, but higher for cured meats compared to before digestion. However, nitrite-cured meats had significantly lower concentrations of all measured aldehydes compared to uncured meats after the duodenal digestion and significantly lower TBARS and hexanal with trends of lower 4-HNE and nonanal after colonic digestion. After duodenal digestion, nitrite-curing resulted in equal TBARS independent of the fat content, while 4-HNE and pentanal were significantly higher in the cured 20% fat meat digest. Compared to their uncured equivalents, 4-HNE formation was only 2.5-fold lower in the 20% fat meat digest, while 12-fold lower concentrations were found in the 5% fat meat digest and no 4-HNE was detected in the 1% fat meat digest.

Because TBARS was the only lipid oxidation parameter that was present in higher concentrations in the colonic as compared to the duodenal digestion, it was hypothesized that TBARS might be present in the human fecal inocula. To investigate this, blank incubations were performed without meat for the duodenal and colonic digestive simulation in a separate run. No aldehydes were detected in the blank duodenal digest, while TBARS concentrations after 72 h of incubation were 5.1 ± 0.1; 17.0 ± 0.2 and 18.3 ± 1.1 nmol/ml digest for the three fecal inocula respectively, indicating a significant contribution of the fecal inoculum to the TBARS levels in the colonic meat digests. One might assume similar effects for the other aldehydes, but these were not measured in the present experimental set up.

Protein oxidation

Increasing PCC concentrations were observed in the following order: undigested < duodenal < colonic digests independent of nitrite-curing (Table 2.3). Fat content did not affect the concentration of PCC before digestion, but nitrite-cured products had lower PCC concentrations than its uncured equivalents before digestion. In the duodenal digests, uncured samples with added fat showed significantly higher PCC concentrations than the ones without added fat. Nitrite-curing led to significantly lower PCC concentrations for the duodenal digests with no significant differences between digests with different fat content. After 72 h of colonic fermentation, a marginal effect of fat content was observed with lower PCC concentrations in the 5% fat meat digests as compared to the other samples.

Table 2.3: Protein oxidation in uncured and nitrite-cured pork containing different amounts of fat (1, 5, 20%) before and after *in vitro* digestion

Nitrite-curing		Uncured			Nitrite-cured				P-Values		
Fat content (%)		**1**	**5**	**20**	**1**	**5**	**20**	**RMSE**	**F**	**NC**	**F×NC**
	Phase										
PCC	BD	1.49	1.74	1.36	1.00	0.76*	1.28	0.261	.838	**< .001**	**.031**
(nmol DNPH/	D	1.84^{b}	2.45^{ab}	2.92^{a}	2.02	1.91	2.22	0.566	**.007**	**.026**	.054
mg protein)	C	4.42	4.10	4.70	4.40	3.49	4.60	0.770	**.035**	.358	.601
Protein	BD	6.70	6.75	6.40	6.60	6.25	5.80	0.304	.227	.120	.580
(mg/ml)	D	2.10	2.15	1.91	1.87	1.83	1.56	0.327	.137	**.011**	.890
	C	1.23^{b}	1.59^{a}	1.23^{b}	1.30^{a}	1.21^{ab*}	0.94^{b}	0.198	**.002**	**.006**	**.022**

Protein carbonyl compounds (PCC) and protein in meat before digestion (BD) (total n=12) were analyzed using a linear model with the fixed effects of fat content (F), nitrite-curing (NC) and their interaction term (F×NC). In duodenal (D) digests (total n=36) and colonic (C) digests (total n=36), these parameters were analyzed using a mixed model with the fixed effects of F, NC and F×NC and the random effect of incubation run. RMSE = root mean square error; a,b,c = means for different fat content (within curing treatment) with different superscripts are significantly different ($P < 0.05$); * = significantly different from uncured equivalent ($P < 0.05$)

Due to digestion, lower protein concentrations were measured in the duodenal and colonic digestive fluids compared to the meat before digestion (Table 2.3). Cured meats had significantly lower protein levels after duodenal and colonic simulation compared to uncured equivalent meats. From the blank incubations, it was estimated that the fecal inocula and SHIME medium contributed for 30-40% of the protein in the colonic fluid. Fecal proteins contained between 5.0 and 8.5 nmol DNPH/mg protein and hence largely contributed to the higher PCC concentrations in the meat colonic digests.

O^6-Carboxymethylguanine and O^6-methylguanine

No O^6-CMG was detected in meats prior to digestion or at the end of the duodenal digestive simulation (Table 2.4). After mimicking colonic digestion, detection was highly dependent on the applied fecal inoculum. Colonic incubation with the inoculum from volunteer 1 did not result in detectable O^6-CMG. The fecal inocula from volunteers 2 and 3 resulted however in low and very high levels of O^6-CMG, respectively. A significant influence of fat content was observed, with higher concentrations of O^6-CMG in both cured and uncured samples containing 20% fat compared to samples lower in fat. The effect of nitrite-curing on the O^6-CMG content was not consistent with significantly lower O^6-CMG when using inoculum 2 and higher O^6-CMG when using inoculum 3. The DNA adduct O^6-MG was not detected in any digestive fluid.

In the blank (no meat) incubations, no O^6-CMG was detected when incubated with fecal inoculum 1 but high DNA adduct concentrations were measured when fecal inoculum 2 (101.0 ± 10.6 ng/ml) and 3 (82.8 ± 26.1 ng/ml) were applied. It should be noted that these blank incubations were performed in a separate run and are therefore not fully comparable to the test incubations.

Table 2.4: NOC-derivative DNA adduct formation by uncured and nitrite-cured pork containing different amounts of fat (1, 5, 20%) before and after *in vitro* digestion

Nitrite-curing		Uncured			Nitrite-cured				P-Values		
Fat content (%)		1	5	20	1	5	20	RMSE	F	NC	F×NC
	Phase										
O⁶-CMG	BD	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	-			
(ng/ml)	D	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	-			
	C1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	-			
	C2	46.8^{c}	50.6^{b}	57.8^{a}	48.9^{a}	44.6^{b*}	50.8^{a*}	0.90	**< .001**	**< .001**	**< .001**
	C3	441^{b}	486^{b}	697^{a}	544^{b}	541^{b}	783^{a}	55.2	**< .001**	**.043**	.824

O⁶-carboxymethylguanine (O⁶-CMG) was not detected (*nd*) in meat before digestion (BD) (total n=12) and after duodenal (D) digestion (total n=36). O⁶-CMG in colonic (C1, C2, C3) digests were analyzed separately (total n=12 per fecal inoculum), due to the very high variation between fecal inocula, using a linear model with the fixed effects of fat content (F), nitrite-curing (NC) and their interaction term (F×NC). RMSE = root mean square error; a,b,c = means for different fat content (within curing treatment) with different superscripts are significantly different ($P < 0.05$); * = significantly different from uncured equivalent ($P < 0.05$)

Short chain fatty acids and NH_3

Since the blank (no meat) incubations resulted in higher O^6-CMG formation compared to the meat digests when the second fecal inoculum was used, we wondered if this might be caused by differences in the fermentation process. Therefore, we repeated 3 blank incubations and 3 digestions of uncured pork containing 5% fat, using fecal inoculum 2. There was no detection of O^6-CMG in the blank or meat digests just after addition of the fecal inoculum. After 72 h of fermentation, the blank incubations contained 118.6 ± 26.2 ng O^6-CMG/ml whereas the meat digests contained only 56.5 ± 4.9 ng O^6-CMG/ml, in line with our previous findings. Significantly higher concentrations of acetate (56.7 ± 9.0 μmol/ml vs. 33.1 ± 0.6 μmol/ml), propionate (24.7 ± 0.7 vs. 14.9 ± 0.3 μmol/ml), butyrate (10.6 ± 1.0 vs. 3.4 ± 0.4 μmol/ml) and NH_3 (34.2 ± 3.1 vs. 18.7 ± 0.8 μmol/ml) were found in the meat digests, compared to the blank digests. Repeated incubations with fecal inoculum 3 confirmed these observations (data not shown).

DISCUSSION

The present *in vitro* digestion study using pork model products allowed to investigate the effects of crucial factors that reflect the variability in composition and properties of meat products, i.e. nitrite-curing and fat content, on oxidation and nitrosation processes during digestion. By adding known pro-oxidants and antioxidants to the applied digestive juices, the *in vivo* situation was mimicked as close as possible. As expected, higher fat contents enhanced the formation of oxidation products and the NOC-derivative DNA adduct O^6-CMG during digestion. Nitrite-curing on the other hand resulted in clearly lower concentrations of oxidation products before, during and at the end of the *in vitro* digestion, whereas the effect on the formation of O^6-CMG proved subjective for inter-individual variation. Previously, Corpet (2011) suggested that the formation of oxidation products and NOCs are amongst those that could explain for the association between high red (processed) meat consumption and CRC.

In accordance to Hur et al. (2009) who used a similar *in vitro* digestion model for beef patties, we observed a significant increase in TBARS concentrations throughout the duodenal digestive simulation. Previously, it was reported that MDA in the small intestine is absorbed into the blood stream (Gorelik et al., 2008b) after which it can reach several organs. Free MDA could react with DNA, resulting in the formation of the MDA-specific DNA adduct pyrimido[1,2-α]purine-10(3H)-one-2'-deoxyribose (M1dG) (Nair et al., 2007). In contrast to TBARS, highest concentrations of 4-HNE were observed in meat before digestion, after which they decreased throughout the digestion. After subtraction of the TBARS values present in the applied fecal inocula, also TBARS concentrations decreased from the duodenal to the colonic step. This decrease of TBARS and 4-HNE during the colonic simulation could be due to reaction with proteins or with bacterial DNA leading to DNA-adducts (Nair et al., 2007).

The antioxidative properties of nitrite were already very clear before digestion since TBARS, 4-HNE, volatile simple aldehydes and PCC were clearly lower or even undetected in cured meats compared to uncured meats. Nitrite is a known antioxidant trough oxygen sequestration and may act as a precursor of the heat-stable NO-myoglobin by which the release of Fe^{2+} during heating is inhibited (Honikel, 2008). Consequently, less Fe^{2+} is available to catalyze the Fenton reaction, which is responsible for the initiation of oxidation processes. Since all meats were sampled fresh and oxidation in fresh meat is generally negligible, the higher lipid aldehyde and PCC concentrations in uncured meats must originate from processing (mincing, adding fat, heating). The clearly lower residual nitrite, found in the nitrite-cured meat samples when higher amounts of subcutaneous pork fat were added, could be explained by interaction of nitrite with unsaturated FA (Trostchansky et al., 2008). Addition of subcutaneous pork fat to meat samples resulted in an expected higher concentration of LOP in meat, since more substrate is available for oxidation. Except for 4-HNE, the expected differences in LOP between the 5 and 20% fat meat samples throughout digestion were not observed. Fat content of grounded beef varying between 10, 15 and 20% also had little effect on lipid oxidation parameters in the meat (Ismail et al., 2009).

The lower values of oxidation products of nitrite-cured meats compared to uncured equivalents in duodenal and colonic fluids are in agreement with recently published work by Chenni et al. (2013) who found that intake of nitrite through drinking water (1 g/l) reduced (25%) haem-induced lipid peroxidation in the colon of rats. This effect was not observed to be significant at lower doses (0.17 g/l nitrite and 0.23 g/l nitrate). Nitric oxide (·NO) is able to react with ROS generating peroxynitrite, which can induce lipid peroxidation. However, ·NO can also react with lipophilic peroxyl radicals, generating far more stable alkyl peroxynitrates. Consequently, it was concluded that a 1:1 ratio of ·NO to ROS enhances lipid peroxidation, while an excess of ·NO results in

inhibition (Darley-Usmar et al., 1995). The reaction between nitrite and unsaturated FA leads to lower residual nitrite levels in the 20% fat meat samples and hence could modify the NO:ROS ratio and induce lipid oxidation. This could explain the higher HNE, pentanal and hexanal concentrations in the cured 20% fat meat sample compared to the cured 1 and 5% fat meat samples.

Also increased protein oxidation was observed throughout the digestion of the meat samples. The higher PCC concentrations after the colonic digestive simulation could be largely attributed to proteins in the fecal inoculum and SHIME medium. No significant differences in PCC concentrations according to fat content were noticed before digestion. It was previously described that lipid radicals may damage proteins by the binding of lipid components such as TBARS and 4-HNE to the protein (Dean et al., 1997). This could explain the higher PCC concentrations in the uncured 20% fat treatment after duodenum digestion. The lower PCC concentrations in cured meat samples before and after duodenal digestive simulations correspond with the associated lower concentrations of LOP.

Next to higher PCC formation, we also observed a significantly higher amount of residual protein in uncured samples, indicating lower protein digestion. These findings agree with Santé-Lhoutellier et al. (2008) who found a highly significant negative correlation between PCC concentrations and proteolytic susceptibility to pepsin. As mentioned before, 4-HNE can also react with proteins through Michael-addition and Schiff base formation, which are known to play a role in protein aggregation. Previously, it was shown that a higher amount of proteins reaching the colonic fermentation, resulted in a higher formation of potentially toxic protein fermentation products such as ammonium, phenol, *p*-cresol and indol. However, higher formation of these products was not associated with enhanced colon cancer promotion in rats (Corpet et al., 1995).

Previously, it was reported that haem-Fe is responsible for endogenous intestinal *N*-nitrosation (Cross et al., 2003). Our results demonstrate that the fat content in meat products enhances the haem-induced intestinal *N*-nitrosation by detecting higher concentrations of the NOC-derivative DNA adduct O^6-CMG when a higher fat content was present in the digested meat. Salivary nitrite or nitrite-cured meats are sources for ·NO formation. Both ·NO and O_2 preferentially diffuse in a lipid environment resulting in accumulation of both reactants in the lipid fraction to form nitrosating species. The rate of this reaction was described to be 300 times faster in a lipid compared to an aqueous environment (Liu et al., 1998), which could explain the facilitating role of fat in the formation of NOCs.

However, the effect of nitrite-curing of meat on O^6-CMG formation was inconsistent in our results, being either promoting or protective depending on the applied fecal inoculum. Possibly, the effect of nitrite-curing depends on the incubation conditions (e.g. O_2 tension), the bacterial composition of the fecal inoculum and interactions with other dietary compounds or metabolites formed during fermentation. This certainly warrants further investigation. However, the effect of nitrite-curing was perceived to be minor compared to the promoting effect of fat content for the meat products examined in the present study.

The formation of the NOC-derivative DNA adduct O^6-CMG was also highly dependent on the applied fecal inoculum. A difference in total anaerobic bacteria in the colonic fluids, fermentation rate or bacterial composition of the used inocula could explain the differences in the magnitude of detection. More research is required to elucidate the underlying mechanisms causing these differences. When O^6-CMG was measured in the colonic digests, blank incubations without meat displayed high values after 72 h of fermentation. Since no O^6-CMG was detected just after addition of the fecal inoculum, its formation must have occurred during the fermentation. The sometimes higher values for the blank incubations than the meat digests is surprising since one expects a

higher fermentation rate and more bacterial growth in the meat digests due to the higher supply of nutrients (protein, glycogen, B vitamins,…). Indeed, meat digests contained approximately 2-fold higher concentrations of NH_3, acetate and propionate, and 3-fold higher concentrations of butyrate compared to the blank digests. However, it has been shown in rats on a high protein diet that high butyrate concentrations in the colon decreased DNA damage in the colonocytes (Bajka et al., 2008). Butyrate was also able to decrease H_2O_2-induced DNA damage in human colonocytes (Rosignoli et al., 2001). Therefore, it seems likely that the 3-fold higher butyrate production during fermentation of the meat digests interfered in the genotoxic activity of compounds. The values of O^6-CMG in the blank digests can thus not be simply subtracted from the values of the meat digests.

In conclusion, our results indicate that addition of fat favors the formation of oxidation products and the NOC-derivative DNA adduct O^6-CMG during the digestion of pork. Nitrite-curing of meat inhibited lipid and protein oxidation but this inhibition was less pronounced throughout digestion when meat in the incubation fluids contained 20% fat. The effect of nitrite-curing on O^6-CMG formation was contradictory in our results and requires more research. The measurement of O^6-CMG was highly variable between fecal inocula originating from different volunteers. In this research, we simulated the passage of meat through the gastro-intestinal tract as close as possible to the *in vivo* situation using static *in vitro* incubations. Several *in vivo* experiments have been conducted towards this subject but these studies do not always gain insights in some mechanistic pathways as the presented *in vitro* work does. However, since the *in vivo* situation is always more complex than *in vitro* conditions, the outcomes in this study should be confirmed *in vivo*.

Chapter III

Increased oxidative and nitrosative reactions during digestion could contribute to the association between well-done red meat consumption and colorectal cancer

Adapted from:

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ABSTRACT

Uncured and nitrite-cured pork were subjected, raw, cooked (65°C, 15 min) or overcooked (90°C, 30 min), to an *in vitro* digestion model, which includes mouth, stomach, duodenum, and colon phases. Heating of uncured meat resulted in a pronounced increase in lipid and protein oxidation products throughout digestion. Nitrite-curing had an antioxidant effect during digestion, but this effect disappeared when the meat was overcooked, resulting in up to nine-fold higher 4-HNE concentrations compared with digested nitrite-cured raw and cooked pork. Colonic digests contained significantly higher concentrations of the NOC-derivative DNA adduct O^6-CMG when pork underwent a more intense heating procedure, independent of nitrite-curing, depending strongly on the fecal inoculum used. Since processed meats are usually nitrite-cured, the present study suggests that overcooking processed meat is likely to result in the formation of genotoxic compounds during digestion and should, therefore, be avoided.

INTRODUCTION

Different independent meta-analyses have demonstrated a significant epidemiological association between CRC risk and high consumption of red (processed) meat (Chan et al., 2011). Moreover, evidence for increased CRC risk has been reported in several epidemiologic studies when the meats consumed have undergone a more intense heating process, such as well-done, fried, barbequed or grilled (Martínez et al., 2007; Rohrmann et al., 2007; Sinha et al., 1999). However, this association was not found in other studies (Tabatabaei et al., 2011; Ollberding et al., 2012). The association between consumption of well-done red meat and CRC is usually explained by the formation of carcinogenic heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs). Santarelli et al. (2008) questioned the importance of these compounds as major

determinants of CRC risk because chicken is a major contributor to HCA (Skog et al., 1997) and PAH intake (Kazerouni et al., 2001), but chicken consumption is not associated with an increased CRC risk in epidemiological studies. Furthermore, Santarelli et al. (2008) argued the dose to induce carcinogenic effects in rodents is 1000- to 100 000-times higher than the dose to which humans are normally exposed by consuming cooked meats. Finally, next to meat and meat products, other food items such as bread, cereals and grains also contribute largely to the dietary intake of total and carcinogenic PAHs (Kazerouni et al., 2001; Falcó et al., 2003; Martí-Cid et al., 2008), while their consumption is not associated with an increased CRC risk. These inconsistencies indicate that other pathways might contribute to the epidemiological association between consumption of well-done red meat and CRC risk. To date, the formation of cyto- and genotoxic LOP, such as MDA and 4-HNE, and NOCs catalyzed by heme-Fe during digestion, are considered to be the most plausible factors contributing to increased risk of developing CRC (Corpet, 2011). A high consumption of red meat by humans has been shown to enhance NOC formation, which is associated with increased colonic formation of the NOC-derivative DNA adduct O^6-CMG (Lewin et al., 2006).

Heating of meat can induce a series of reactions that are able to enhance lipid and protein oxidation, including inactivation of antioxidant enzymes (e.g glutathion peroxidase), an increase in free Fe^{2+} through destruction of the heme-porphyrin moiety, and the release of O_2 from oxymyoglobin with production of H_2O_2 (Kanner, 1994). Nitrite-curing of meat is a common meat processing procedure that induces color formation, inhibits growth of *Clostridium botulinum* and delays oxidative rancidity. Among other antioxidant mechanisms, nitrite may act as a precursor of the heat-stable NO-heme, through which the release of Fe^{2+} during heating is inhibited. Because of this, nitrite-curing interacts with the destruction of heme-Fe during heating of meat and, hence, an effect on the oxidation and nitrosation pathway during digestion is

suspected. Thus, we aimed to investigate the modulating effects of heating and nitrite-curing of meat on markers of lipid oxidation (TBARS, 4-HNE, simple aldehydes) and protein oxidation (PCC) during *in vitro* digestion. Many NOCs are alkylating agents that can induce carboxymethylation of DNA. Therefore, we also measured formation of the NOC-derivative DNA adduct O^6-CMG during *in vitro* digestion. Previously, using the same *in vitro* approach, we demonstrated the role of heme-Fe and fat content on oxidation processes and O^6-CMG formation during the digestion of meat as well as the inhibitory effect of nitrite-curing on oxidation (with no effect on O^6-CMG formation) (Vanden Bussche et al., 2014; Chapter I and II).

MATERIALS AND METHODS

Experimental Setup

Uncured and nitrite-cured pork products, containing 5% total fat, were subjected, raw, cooked (65°C, 15 min) or overcooked (90°C, 30 min), to an *in vitro* digestion model. Each incubation run included all treatments in quadruplicate from which 2 replicates underwent digestion as far as the duodenum and 2 until the colon. Each incubation was performed three times with fecal inoculum (FI) originating from three different individuals.

Preparation of the Meat Samples

Commercially available lean meat samples from porcine m. *Longissimus dorsi* were purchased at the local supermarket. The loin was chopped manually into cubes of approximately 1-2 cm^3. Subcutaneous pork fat from one batch was added to the chopped meat to obtain a targeted total

fat content of 5%. Meat samples with added fat were first ground using a grinder (Omega T-12) equipped with a 10 mm plate and, subsequently, a 3.5 mm plate. After grinding, the meat was cured by adding 20 g of 0.6% nitrite salt/kg meat, corresponding to an added concentration of 120 mg of nitrite/ kg meat. Next, the samples were heated in a warm water bath for 15 min after the core temperature had reached 65°C (cooked) or for 30 minutes after the core temperature had reached 90°C (overcooked). Raw and heated meat samples were homogenized in three 5 s bursts using a food processor (Moulinex DP700), vacuum packed and stored at -20°C until the start of the incubation experiments.

Digestive simulations

See Chapter I (p. 21).

Preparation of Human Fecal Inoculum

See Chapter I (p. 23).

Chemical Composition of the Meat Samples

See Chapter I (p. 25).

Oxidation Products

See Chapter I (p. 25).

O^6-Carboxymethylguanine

See Chapter I (p. 27).

Short chain fatty acids and NH_3

See Chapter II (p. 45).

Statistical Analysis

For the meat characteristics, a linear model ANOVA procedure (SAS enterprise guide 5) was used with the fixed effects of heating conditions, nitrite and heating conditions × nitrite. Data on TBARS, 4-HNE, simple aldehydes, PCC and protein content were analyzed for the undigested meat, and the duodenum and colon steps separately. For the undigested meat, the same linear model as for meat characteristics was used, while for the duodenum and colon phases, a mixed model ANOVA procedure was used, with the fixed effects heating conditions, nitrite and heating conditions × nitrite and the incubation run as a random effect. Data on O^6-CMG were analyzed using a linear model, with the fixed effects heating conditions, nitrite and heating conditions × nitrite. Tukey-adjusted *post hoc* tests were performed for all pairwise comparisons with $P<0.05$ considered significant.

RESULTS

Meat Characteristics

Table 3.1 shows the composition of the pork model products used in the *in vitro* digestions. Dry matter and fat content significantly increased with a higher intensity of heating due to water evaporation. More intense heating conditions resulted in lower proportions of MUFA, total PUFA and LC n-3 PUFAs in the FA profile. Residual nitrite in the overcooked nitrite-cured pork was significantly lower than in the raw and cooked nitrite-cured pork. Residual nitrite was 31.8, 30.3 and 13.2% of the amount added to the raw, cooked and overcooked pork, respectively. Despite originating from the same pork batch, nitrite-cured meats contained significantly less total Fe and more heme-Fe than uncured samples.

Table 3.1: Composition of the pork model products used in the *in vitro* digestions

Nitrite-curing		Uncured			Nitrite-cured				P-values		
Heating conditions		R	C	OC	R	C	OC	RMSE	HC	NC	HC×NC
Dry matter	%	29.6^{b}	31.9^{a}	32.3^{a}	30.3^{b}	31.1^{ab}	32.4^{a}	1.23	**< .001**	.932	.162
Protein	%	21.6	21.5	23.1	21.3	22.2	23.4	1.19	.143	.682	.837
Fat	%	4.9	5.6	5.8	5.0	5.4	5.5	0.37	**.008**	.257	.560
SFA	g/100g FAME	38.2	38.0	37.6	37.9	38.3	37.5	0.467	.205	.817	.641
MUFA	g/100g FAME	42.7	42.2	41.2	42.1	42.4	41.2	0.560	**.039**	.582	.664
PUFA	g/100g FAME	16.2^{a}	16.0^{ab}	15.5^{b}	15.7	15.9	15.7	0.190	**.041**	.188	.084
ALA	g/100g FAME	0.68	0.69	0.65	0.64	0.65	0.66	0.037	.879	.463	.630
LC *n*-3 PUFA	g/100g FAME	0.31^{a}	0.29^{ab}	0.27^{b}	0.26	0.27	0.25	0.020	**.019**	.197	.067
LA	g/100g FAME	13.1	12.9	12.6	12.8	12.8	12.7	0.175	.068	.360	.303
LC *n*-6 PUFA	g/100g FAME	1.38^{a}	1.36^{b}	1.34^{b}	1.28*	1.38	1.36	0.021	.098	.183	**.010**
Residual nitrite	mg/100g	-	-	-	3.81^{a}	3.64^{a}	1.58^{b}	0.799	**< .001**	-	-
Total Fe	mg/100g	0.45^{b}	0.55^{ab}	0.77^{a}	0.42	0.49	0.49	0.121	**.024**	**.024**	.092
Heme-Fe	mg/100g	0.26	0.25	0.21	0.26	0.31	0.33	0.022	.139	**.006**	**.027**

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; ALA = α-linolenic acid (C18:3, *n*-3); LC *n*-3 PUFA = long chain omega-3 polyunsaturated fatty acids (C20:5, *n*-3; C22:5, *n*-3; C22-6, *n*-3); LA = linoleic acid (C18:2, *n*-6); LC *n*-6 PUFA = long chain omega-6 polyunsaturated fatty acids (C20:4, *n*-6; C22:4, *n*-6; C22:5, *n*-6); RMSE = root mean square error; R = raw; C = cooked (65°C, 15min); OC = overcooked (90°C, 30min); HC = heating conditions; NC = nitrite-curing; a,b,c = means for different heating conditions (within curing treatment) with different superscripts are significantly different ($P < 0.05$); * = significantly different from uncured equivalent ($P < 0.05$).

SCFA and NH_3

Fermentation with FI.3 resulted in significantly lower concentrations of NH_3, acetate, propionate and butyrate compared with the other FIs (Figure 3.1), while FI.1 resulted in significantly lower concentrations of NH_3 and butyrate, but higher concentrations of acetate compared with FI.2.

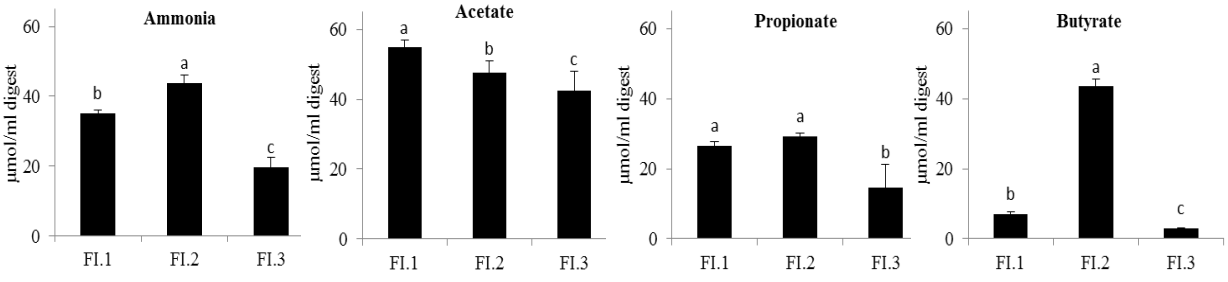

Figure 3.1: concentrations of ammonia and the short chain fatty acids acetate, propionate and butyrate in the colonic juices of the applied fecal inocula (FI) originating from 3 individuals (FI.1, FI.2 and FI.3) (n=12 per fecal inoculum). a,b,c = means for different fecal inocula with different superscripts are significantly different ($P < 0.05$).

Lipid Oxidation

Before digestion, uncured cooked and overcooked pork displayed significantly higher TBARS and 4-HNE concentrations than uncured raw samples, while the uncured cooked pork contained significantly more pentanal, hexanal and heptanal compared with the overcooked and raw samples (Table 3.2). All LOP were significantly lower when the pork was nitrite-cured with no differences arising from the heating conditions. 4-HNE and heptanal could not be detected in the nitrite-cured pork.

Table 3.2: lipid oxidation in uncured and nitrite-cured raw, cooked and overcooked pork before and after *in vitro* digestion

Nitrite-curing		Uncured			Nitrite-cured				P-Values		
Heating conditions		**R**	**C**	**OC**	**R**	**C**	**OC**	**RMSE**	**HC**	**NC**	**HC×NC**
	Phase										
TBARS	BD	1.7^{b}	5.4^{a}	5.7^{a}	1.9	2.0*	2.0*	0.14	**< .001**	**< .001**	**< .001**
(nmol/mL)	D	9.3^{c}	13.6^{b}	15.2^{a}	8.2^{b*}	8.2^{b*}	9.4^{a*}	0.58	**< .001**	**< .001**	**< .001**
	C	11.7^{c}	15.1^{b}	16.4^{a}	11.3	10.3*	10.6*	0.80	**< .001**	**< .001**	**< .001**
4-HNE	BD	10.3^{b}	221.2^{a}	162.0^{a}	*nd*	*nd*	*nd*	30.14	**.003**		
(pmol/mL)	D	82.8^{b}	203.4^{a}	181.0^{a}	15.2^{b}	18.7^{b*}	166.0^{a}	40.32	**.003**	**.003**	**.046**
	C	27.0	39.4	40.8	34.5^{b}	50.6^{a}	51.3^{a}	8.63	**.016**	**.041**	.935
Pentanal	BD	44^{c}	172^{a}	93^{b}	46	32*	40*	7.6	**< .001**	**< .001**	**< .001**
(pmol/mL)	D	129^{b}	226^{a}	247^{a}	68^{b}	71^{b*}	163^{a*}	35.1	**< .001**	**< .001**	**.008**
	C	114	103	99.1	96	102	101	27.1	.908	.534	.650
Hexanal	BD	89^{c}	1162^{a}	550^{b}	74	76*	78*	33.0	**< .001**	**< .001**	**< .001**
(pmol/mL)	D	303^{b}	696^{a}	704^{a}	120^{b}	134^{b*}	401^{a*}	113.2	**< .001**	**< .001**	**< .001**
	C	149	174	173	150	177	165	15.6	**.001**	.826	.691
Heptanal	BD	*nd*	18.4^{a}	8.8^{b}	*nd*	*nd*	*nd*	2.36	**.021**		
(pmol/mL)	D	20.6^{b}	51.4^{a}	47.4^{a}	9.8^{b}	13.3^{b*}	32.6^{a*}	7.26	**< .001**	**< .001**	**< .001**
	C	65.5	52.2	47.6	63.8	96.9	88.9	27.01	.664	**.004**	.082
Nonanal	BD	8.7	14.9	15.1	8.2	10.3	8.1	3.61	.087	.305	.454
(pmol/mL)	D	15.7^{b}	23.0^{a}	26.3^{a}	13.6^{b}	16.8^{b*}	22.4^{a}	3.12	**< .001**	**< .001**	.288
	C	13.9	13.2	15.0	13.8	15.1	14.5	2.34	.638	.613	.394

TBARS = thiobarbituric acid reactive substances; 4-HNE = 4-hydroxy-2-nonenal; BD = before digestion (total n=12); D = duodenal stage (total n=36); C = colonic stage (total n=36); R = raw; C = cooked (65°C, 15min); OC = overcooked (90°C, 30min); RMSE = root mean square error; HC = heating conditions; NC = Nitrite-curing; a,b,c = means for different cooking methods (within curing treatment) with different superscripts are significantly different ($P < 0.05$); * = significantly different from uncured equivalent ($P < 0.05$); *nd* = not detected.

After duodenal digestion, TBARS were significantly higher in the uncured overcooked pork compared with the uncured cooked pork digests followed by the raw pork digest. Significantly more lipid aldehydes were produced during uncured cooked and overcooked pork digestion compared with raw pork digestion. Significantly lower concentrations of pentanal, hexanal and heptanal, when uncured pork was overcooked compared with cooked, were not observed after digestion. A greater increase in concentrations of TBARS and pentanal was observed during digestion of overcooked uncured pork compared with cooked uncured pork. Similarly, whereas there was a 50% reduction in hexanal concentrations during digestion of the cooked uncured pork, formation of netto hexanal was observed during the digestion of overcooked uncured pork. Even though no differences were observed in the nitrite-cured pork subjected to different heating conditions before digestion, significantly higher concentrations of all lipid aldehydes were observed when the nitrite-cured pork was overcooked compared with the cooked and raw sample. The nitrite-cured pork showed no significant differences compared with its uncured equivalent for 4-HNE and nonanal concentrations after digestion when the digested meat was overcooked, but there was a significant difference when the nitrite-cured pork was cooked. Digests of nitrite-cured meat contained marginally but significantly more TBARS and nonanal, and nine-fold higher 4-HNE, two-fold higher pentanal, three-fold higher hexanal and 2.5-fold higher heptanal concentrations when the meat was overcooked compared with cooked and raw meat.

After 72 h of fermentation, all aldehydes, except TBARS and heptanal, were significantly lower compared with duodenal digesta. The effect of heating and nitrite-curing was still significant for TBARS in colonic digesta. Both factors were also significant for 4-HNE, but the nitrite-cured samples had significantly higher 4-HNE concentrations in the mimicked colon, albeit marginally. Hexanal increased significantly with heating. Similar to 4-HNE, heptanal was significantly higher

in the colonic digested nitrite-cured pork samples. No significant effects on pentanal and nonanal were observed after the mimicked colon digestion.

Protein Oxidation

More intense heating procedures were accompanied by significantly higher protein oxidation in the uncured pork before and after digestion (Table 3.3). Before digestion, nitrite-curing resulted in significantly lower PCC concentrations. After duodenal digestion, digesta of nitrite-cured overcooked pork contained significantly higher PCC concentrations compared with nitrite-cured raw and cooked pork digesta, but this was not observed after colonic digestion.

A clear decrease in protein levels was observed throughout the digestion from stomach to duodenum and through to the colon. A significant effect of nitrite-curing, however, was only observed in the simulated duodenum. Nitrite-cured overcooked samples had significantly less protein compared with uncured overcooked samples, which amounted to only half the protein found in the uncured sample.

Table 3.3: protein oxidation in uncured and nitrite-cured raw, cooked and overcooked before and after *in vitro* digestion

Nitrite-curing		Uncured			Nitrite-cured				P-Values		
Heating conditions		R	C	OC	R	C	OC	RMSE	HC	NC	HC×NC
	Phase										
PCC	BD	0.77^{b}	1.80	2.52^{a}	1.09	0.83^{*}	1.27	0.326	**.032**	**.026**	.065
(nmol DNPH/	D	1.49^{b}	2.09^{b}	2.92^{a}	1.90^{b}	1.52^{b}	3.50^{a*}	0.414	**< .001**	.317	**.004**
mg protein)	C	2.38^{b}	3.73^{a}	3.88^{a}	2.57	3.45	3.18	0.546	**< .001**	.162	0.154
Protein	BD	5.48[1]	6.44	5.89[1]	5.57[1]	6.09	6.12[1]	0.212	-	-	-
(mg/mL)	D	2.69	2.47	2.99	2.32	2.39	1.49^{*}	0.653	.599	**.006**	**.030**
	C	1.31	1.38	1.41	1.53	1.22	1.38	0.295	.912	.608	.293

PCC = protein carbonyl compounds; BD = before digestion (total n=12); D = duodenal stage (total n=36); C = colonic stage (total n=36); R = raw; C = cooked (65°C, 15min); OC = overcooked (90°C, 30min); RMSE = root mean square error; HC = heating conditions; NC = Nitrite-curing; a,b,c = means for different cooking methods (within curing treatment) with different superscripts are significantly different ($P < 0.05$); * = significantly different from uncured equivalent ($P < 0.05$); [1] = only 1 sample analyzed.

O^6-carboxymethylguanine

No detection of O^6-CMG was observed before and after duodenal digestion. In the colonic digesta, detection of O^6-CMG depended strongly on the FI used; O^6-CMG was detected only when FI.3 was used. No significant differences were found between the individual different treatments but, overall, more intense heating significantly increased O^6-CMG concentrations. No significant effect of nitrite-curing was observed (Figure 3.2).

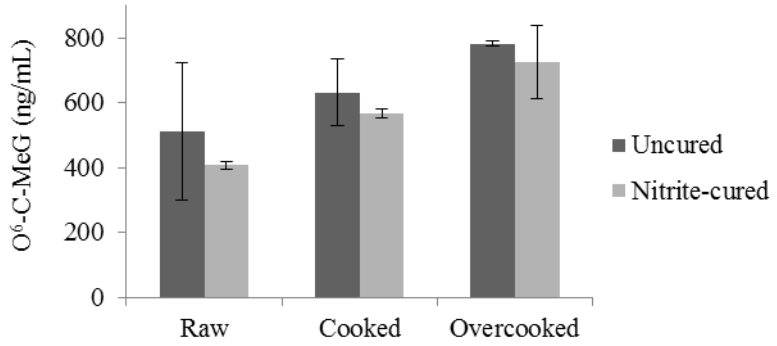

Figure 3.2: O^6-Carboxymethylguanine (O^6-CMG) concentrations (ng/mL) in uncured and nitrite-cured raw, cooked and overcooked pork after *in vitro* colonic digestion using fecal inoculum 3 (total n=12). More intense heating conditions of the pork significantly stimulated O^6-CMG formation (P = .023) while the effect of nitrite-curing was not significant (P = .267). Error bars indicate standard deviations of two replicate determinations.

DISCUSSION

In this *in vitro* digestion study, heating of pork model products stimulated oxidation and O^6-CMG formation, while nitrite-curing inhibited lipid oxidation throughout the digestion with no significant effect on O^6-CMG formation. However, the antioxidant effect of nitrite-curing was far less pronounced if at all when pork was overcooked. The two cooking conditions represent a common heating treatment applied to many fresh or cooked meat products, and a more extreme treatment to which some meat products are subjected accidentally or purposely. For example, an internal temperature of 60-65°C corresponds to 'medium' doneness of cooked beef, and cooked ham is

manufactured by cooking at approximately 70°C for about two hours. The 'overcooked' condition in the present study corresponds to, for example, canned meat products that are sterilized by intense heating or meat products prepared by deep-frying or boiling.

Previously, Corpet (2011) explained the association between consumption of processed meats and CRC risk could be attributed to the formation and activity of cyto- and genotoxic oxidation products, such as MDA and 4-HNE and genotoxic NOCs. The higher epidemiologic risk of CRC with more consumption of meat that has been heated intensively is usually explained by the formation of carcinogenic HCAs and PAHs. One of the most important inconsistencies in this theory is that consumption of chicken, a major source of HCAs, is not associated with increased CRC risk in epidemiological studies. However, increased CRC risk caused by formation of oxidation and nitrosation products would correspond to epidemiological findings since these products are found in low concentrations during digestion of chicken compared with beef or pork (Chapter I). Thus, the formation of these genotoxic products could help explain the association between consumption of intensively heated meat products and increased CRC risk.

Free Fe^{2+} increases considerably during cooking of uncured meats, while nitrite-curing prevents destruction of heme-Fe by stabilizing the porphyrin ring (Chen et al., 1984), as observed in our results. Heating treatment also results in a decrease of antioxidant enzymes, such as glutathione peroxidase (Hoac et al., 2006), and releases oxygen from oxymyoglobin, which leads to production of H_2O_2 (Kanner, 1994). Free Fe^{2+} catalyzes the Fenton reaction through which oxidative processes are initiated. Through their reactive nature, ROS cause oxidative damage to PUFAs and proteins present in meat, explaining the higher formation of TBARS and 4-HNE when uncured pork is

heated. The lower concentrations of simple aldehydes when uncured pork is overcooked, compared with cooked meat, could be explained by evaporation of aldehydes caused by intense heating or decreased pro-oxidant effect of oxymyoglobin when heated to temperatures above 75°C, which has been observed previously (Bou et al., 2008). When meats are nitrite-cured, less destruction of the heat-stable NO-heme may lead to less Fe^{2+} being released to initiate oxidation processes, resulting in significantly lower lipid oxidation.

Because the Fenton reaction is a chain reaction, higher concentrations of oxidation products after digestion were to be expected, as previously demonstrated (Chapter I). When nitrite-cured pork was overcooked, the antioxidant effect of nitrite-curing during digestion was remarkably reduced. This could be explained partly by the lower residual nitrite concentrations observed in the overcooked meat (90°C, 30 min) compared with the raw and cooked meat (65°C, 15 min). In agreement, Okayama et al. (1991) found enhanced decomposition of nitrite, especially with longer cooking time or when temperatures reached 80°C. A 1:1 ratio of nitric oxide (·NO) to ROS stimulates lipid oxidation while ·NO>ROS inhibits this process (Darley-Usmar et al., 1995). The lower residual nitrite caused by more intense heating is likely to modify the ·NO:ROS ratio and, hence, nitrite could shift from antioxidant to pro-oxidant behavior, which might explain the increased formation of oxidation products in the overcooked nitrite-cured samples. Since there was a nine-fold higher formation of genotoxic aldehyde 4-HNE when nitrite-cured pork was overcooked, compared with nitrite-cured cooked and raw pork, more intense heating of processed meats are likely to impact human health negatively. Earlier research by Gorelik et al. (2008) showed that MDA is absorbed into the bloodstream and LOP could reach tissues causing DNA damage (Nair et al., 2007).

Lower amounts of LOP in colonic digests could be attributed to Michael-addition type reactions with proteins, binding to bacterial DNA (Nair et al., 2007), or oxidation by bacterial aldehyde

dehydrogenase activity. An effect of heating on the concentrations of LOP was still present despite the net disappearance of aldehydes during fermentation. The effect of nitrite-curing of meat in the colonic step was remarkable since it was associated with lower TBARS concentrations and marginally increased 4-HNE and doubled heptanal concentrations in the cooked and overcooked samples. Previously, nitrite-curing of pork and beef also resulted in a two-fold difference in heptanal concentrations in simulated colonic digests compared with their uncured equivalents (Chapter I). Thus, this dual role of nitrite-curing on oxidation should be further investigated.

Similar to our results on lipid oxidation, heating of uncured pork has been reported to increase protein oxidation. Bax et al. (2012) found increased PCC concentrations when uncured meat was heated above 100°C, while Gatellier et al., (2010) found no changes in PCC when beef was heated at 65°C or 96°C. In the present study, the overcooked nitrite-cured pork had lower PCC levels than its uncured equivalent before digestion, but a pro-oxidant activity of nitrite in the overcooked pork was observed after duodenal digestion. However, it should be noted that residual protein from the overcooked nitrite-cured pork in the duodenal digestions was only half that of digested overcooked uncured pork. Hence, the total amount of oxidized protein in the digests of the overcooked nitrite-cured pork was still less than the digesta of overcooked uncured pork. Previously, it has been reported that high PCC values are associated with a lower protein digestibility (Santé-Lhoutellier et al., 2008), as also observed in our previous *in vitro* digestion study (Chapter I) Lipid aldehydes, such as MDA and 4-HNE, are able to react with protein chains contributing to protein aggregation making the protein less susceptible to pepsin activity. Since the overcooked nitrite-cured pork had low amounts of PCC, and LOP before digestion, it is likely that the meat proteins were initially well digested in the stomach, after which the low amounts of residual protein reacted with an increasing amount of MDA and 4-HNE, which were formed in a later phase of the digestion. The rate of

protein digestibility could be of importance in relation to CRC since higher amounts of residual protein reaching the colon could lead to the formation of potentially harmful protein fermentation products, such as ammonia, phenol, *p*-cresol and indol (Windey et al., 2012).

Next to the higher formation of oxidation products during digestion of the heated meat products, this study also demonstrated greater formation of the NOC-derivative DNA adduct O^6-CMG during colonic digestion when meats underwent a more intense heating. Previously, it was described that NOCs can be activated either enzymatically or non-enzymatically through oxidation (Miura et al., 2011; Tsutsumi et al., 2010). This non-enzymatic activation of NOCs can be caused by a hydroxyl radical generating system, consisting of Fe^{2+}, Cu^{2+}, ascorbic acid and H_2O_2. These are all compounds present in meat or the applied digestive juices. When Fe^{2+}, Cu^{2+} or H_2O_2 were removed from a reaction mixture with *N*-nitroso-*N*-methylpentylamine, the mutagenicity of the mixture decreased (Miura et al., 2011). Thus, we hypothesize that higher concentrations of free Fe^{2+} or higher rates of oxidation during digestion of heated meat might contribute to a greater activation and mutagenicity of NOCs and, hence, induce more of the NOC-derivative DNA adduct O^6-CMG, as observed in our study. Furthermore, early research by Dix & Lawrence (1983) showed that oxidizing agents generated during lipid oxidation are able to epoxidize a common PAH, benzo[α]pyrene, to its carcinogenic form. This could contribute to explaining why consumption of chicken, despite containing considerable amounts of HCAs and PAHs, is not associated with a higher risk of CRC in epidemiological studies since the extent of oxidation during digestion is very low compared with pork and beef products (Chapter I).

Nitrite-curing of meat had no significant effect on O^6-CMG formation, but a possible influence of nitrite-curing cannot be ruled out. Santarelli et al. (2010) found higher *mucin depleted foci* as a marker for pre-neoplastic lesions in the colon of rats fed oxidized nitrite-cured dark meat. However, this

was not found in rats fed anaerobically packed nitrite-cured dark meat, which is similar to our meat. Possibly, a combination of nitrite-curing and oxidation is required to induce increased nitrosation reactions.

It is unclear why only one of the three FI used resulted in a high O^6-CMG formation. Recently, Vanden Bussche et al. (2014) reported a high variation in O^6-CMG formation between 15 FI used during *in vitro* digestion of red meat. The underlying cause might involve differences in individual microbial gut populations, fermentation rates or the presence of other dietary compounds in the FI. It is remarkable that the FI resulting in the lowest SCFA concentrations was associated with greater O^6-CMG formation since SCFA, such as butyrate, are described as protective against CRC development (Hamer et al., 2008). Acetate and butyrate are also thought to inhibit oxidative DNA-damage (Abrahamse et al., 1999). Future experiments should clarify this pronounced effect of FI on O^6-CMG formation.

Since the present data showed increased formation of oxidation products and O^6-CMG during digestion of meat samples that underwent a more intense heating, future studies investigating the association between cooking methods and CRC should also focus on these pathways. Increased CRC risk caused by consumption of well-done red meat is usually explained by the formation of HCAs and PAHs, but this hypothesis is not consistent with epidemiological findings. Therefore, we suggest the formation of NOCs and oxidation products might be more relevant. Nitrite-curing had an antioxidant effect during digestion, but this effect disappeared when the meat was overcooked, resulting in increased concentrations of all aldehydes compared with digested nitrite-cured raw and cooked pork. Since processed meats are usually nitrite-cured, the present study suggests that overcooking processed meat is likely to result in the formation of genotoxic compounds during digestion and should, therefore, be avoided.

Chapter IV

Reducing compounds equivocally influence oxidation during digestion of a high-fat beef product, which promotes cytotoxicity in colorectal carcinoma cell lines

Adapted from:

Van Hecke, T., Wouters, A., Rombouts, C., Izzati, T., Berardo, A., Vossen, E., Claeys, E., Van Camp, J., Raes, K., Vanhaecke, L., Peeters, M., De Vos, W.H., De Smet, S. (2016). Reducing Compounds Equivocally Influence Oxidation during Digestion of a High-fat Beef Product, which Promotes Cytotoxicity in Colorectal Carcinoma Cell Lines. *Journal of Agricultural and Food Chemistry*, 64(7), 1600–1609.

ABSTRACT

We studied the formation of TBARS, 4-HNE and hexanal during *in vitro* digestion of a cooked low-fat and high-fat beef product in response to the addition of reducing compounds. We also investigated whether higher LOP in the digests resulted in a higher cyto- and genotoxicity in Caco-2, HT-29 and HCT-116 cell lines. High-fat compared to low-fat beef digests contained approximately 10-fold higher LOP concentrations (all $P<0.001$), and induced higher cytotoxicity ($P<0.001$). During digestion of the high-fat product, phenolic acids (gallic, ferulic, chlorogenic and caffeic acid) displayed either pro-oxidant or antioxidant behavior at lower and higher doses respectively, whereas ascorbic acid was pro-oxidant at all doses, and the lipophilic reducing compounds (α-tocopherol, quercetin and silibinin) all exerted a clear antioxidant effect. During digestion of the low-fat product, the hydrophilic compounds and quercetin were antioxidant. Decreases or increases in LOP concentrations amounted to 100% change versus controls.

INTRODUCTION

Recent meta-analyses have reported an elevated risk for developing CRC (+18%), coronary heart disease (+42%) and diabetes mellitus (+19%) per 50g of processed meat consumption per day (Chan et al., 2011; Micha et al., 2010). Oxidative stress might be a common factor contributing to the onset and/or progression of these diseases. Previously, we have demonstrated the increased formation of the cyto- and genotoxic LOP such as MDA, 4-HNE and hexanal (HEX) in an in *vitro* digestion protocol, when processed meat contained a high haem-Fe content (Chapter I), high fat content (Chapter II) or was heated (Chapter III), compared to their respective controls. Gorelik et al. (2008) showed MDA was absorbed in humans following consumption of turkey cutlets, after which it was able to modify low-density lipoproteins (LDL) (Gorelik et al., 2013),

which may trigger the onset and progression of atherosclerosis. Strategies to inhibit these oxidative processes or scavenge their products during digestion of processed meat could mitigate these health risks. Previously, it was shown that oxidation during digestion can be reduced when red meat is combined with other foods and compounds such as capers (Tesoriere et al., 2007), red wine polyphenols (Gorelik et al., 2008b), or coffee melanoids (Tagliazucchi et al., 2010).

Meat is a complex matrix containing fat, proteins, free and bound iron, which are all compounds involved in oxidation processes. During the self-maintaining Fenton reaction, Fe^{2+} is oxidized to Fe^{3+} and reduced back to Fe^{2+} catalyzed by H_2O_2, producing a hydroxyl radical ($HO\cdot$) and a hydroperoxyl radical ($HOO\cdot$). These ROS are highly unstable and initiate a chain of oxidative reactions, leading to the formation of various lipid and protein oxidation products. During gastro-duodenal digestion, meat is exposed to the acidic environment of the stomach, which favors lipid oxidation (Kanner et al., 2001), and to a variety of anti- and pro-oxidants present in the digestive juices such as nitrite in saliva (Volk et al., 2009) and ascorbic acid (Dabrowska-Ufniarz et al., 2002) in gastric juice. Furthermore, many processed meats have a considerable fat content, which is emulsified in the small intestine by lipases and bile salts (McClements and Li, 2010). The reaction mechanism of oxidation is considerably different among aqueous solutions, bulk fat or emulsions (McClements and Decker, 2000). Therefore, the evaluation of the antioxidant activity of reducing compounds during digestion should take these physico-chemical aspects into account.

In this study, we aimed at elucidating whether dietary reducing compounds could alleviate oxidative reactions during the digestion of processed meat. For this purpose, we tested the addition of a set of reducing compounds during the *in vitro* digestion of a low-fat and high-fat beef product. These compounds differed in solubility and included (i) the lipophilic compounds

α-tocopherol, quercetin and silibinin and, (ii) the hydrophilic compounds ascorbic acid, gallic acid, caffeic acid, ferulic acid and chlorogenic acid. We hypothesized that lipophilic reducing compounds may exert more efficient antioxidant activity than hydrophilic compounds during digestion of meat products, in view of their partition in the fat compartment and may protect PUFAs more efficiently from oxidative processes.

Secondly, we investigated whether the inhibition of LOP formation by selected compounds resulted in a lower cyto- and genotoxicity when human colorectal carcinoma cell lines were exposed to the meat digests. Previously, the cyto- and genotoxic effects of pure meat-related compounds such as hemin and hemoglobin (Glei et al., 2006; Klenow et al., 2009), and of fecal water from rats fed a high-meat diet supplemented with various additives (Pierre et al., 2013) have been described. The application of *in vitro* digests on cell lines might be a useful tool to study the cyto- and genotoxic potential of various meat digests. This strategy was previously applied, using digested herb-enriched beef patties (Ryan et al., 2009; Daly et al., 2010) and other *in vitro* digested foods such as nuts (Lux et al., 2012), bread (Schlörmann et al., 2012), or tomatoes (Palozza et al., 2007).

MATERIALS AND METHODS

Chemicals

The reducing compounds α-tocopherol (>96% purity; T3251), gallic acid (>97.5% purity; G7384), ascorbic acid (>95% purity; A0278), caffeic acid (> 98% purity, C0625), chlorogenic acid (>95% purity, C3878), ferulic acid (99% purity; 128708), quercetin aglycone (>95% purity, Q4951) and silibinin (>98% purity, S0417) were purchased from Sigma Aldrich (Diegem, Belgium).

Manufacturing of meat samples

Lean meat samples from the *m. Pectoralis profundus* of beef were collected in a local slaughterhouse. The meat was manually chopped into cubes of approximately 1-2 cm^3. A high-fat beef product was obtained by adding pig subcutaneous fat to the muscle to an estimated total fat content of 15%. Both the low-fat and the high-fat beef products were minced in a grinder (Omega T-12) equipped with a 10 mm plate, followed by grinding through a 3.5 mm plate. Next, the meat samples were vacuum packed in plastic bags and heated in a warm water bath for 15 min after the core temperature had reached 65°C. After manufacturing, all meat samples were homogenized in two 5 s bursts using a food processor (Moulinex DP700), vacuum packed and stored at -20°C until the start of the incubation.

Digestive simulations

The *in vitro* digestions of the beef products with the active compounds were performed according to a previously described protocol, specific for studying oxidation processes during passage in the gastrointestinal system (Chapter I). Digestions consisted of an enzymatic digestion simulating the mouth, stomach and duodenum gastro-intestinal tract compartments. In addition to the previously described composition (Chapter I), amylase (290 mg/L) was added to the saliva (Versantfoort et al., 2005). Digestions of the low-fat and high-fat beef product were performed in duplicate with either 0, 2.5, 5, 10 or 20 mg of ascorbic acid, caffeic acid, ferulic acid, chlorogenic acid, gallic acid, α-tocopherol, quercetin or silibinin. The reducing compounds were dissolved in water (except for α-tocopherol which was added as such), and 1 mL was added to the incubation jar with the prepared meat samples (4.5 g) at the start of each incubation. The incubations were performed in duplicate at 37°C for 5 minutes with 6 mL saliva, 2 hours with 12 mL gastric juice, and 2 hours with 2 mL bicarbonate buffer (1 M, pH 8.0), 12 mL duodenal juice and 6 mL bile

juice. After completion, samples were homogenized with an ultraturrax (9500 rpm) and aliquots were stored at -80°C for further analysis.

Chemical composition of the meat samples

See Chapter I (p. 25).

Oxidation products

See Chapter I (p 25).

Cell culture

Based on the results from the *in vitro* biochemical assays, 4 treatments were chosen for use in cyto- and genotoxicity assays in triplicate: low-fat beef product (low in LOP formation), high-fat beef product (high in LOP formation), high-fat beef product + 5mg ascorbic acid (stimulation of LOP formation) and high-fat beef product + 5mg quercetin (inhibition of LOP formation).

Three human colorectal carcinoma cell lines (Caco-2, HT-29 and HCT-116), obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were used. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. All cell culture reagents were purchased from Life Technologies (Ghent, Belgium). Cell lines were maintained at 37°C and 5% CO_2/95% air in a humidified incubator and confirmed free of mycoplasma infection through regular testing (MycoAlert Mycoplasma Detection Kit, Lonza, Verviers, Belgium).

Real-time monitoring of the cytotoxicity of meat digests by xCelligence (RTCA)

Cell proliferation of Caco-2, HT-29 and HCT-116 cells was continuously monitored in 15 min intervals for a total time of 96 h at a temperature of 37°C by using modified 16-well E-plates and the xCelligence RTCA DP instrument (Westburg, Leusden, The Netherlands). As described previously (Limame et al., 2012), background impedance signal was measured following 30 min incubation with 100 µL DMEM cell culture growth medium per well. Next, 50 µL cell suspension (1000 cells/well for Caco-2 and HCT-116; 2000 cells/well for HT-29) was seeded into the wells. The impedance value of each well was monitored automatically by the xCelligence system and expressed as a cell index (CI) value. 24 h after seeding, cells were treated for 72 h with 30 µL of a 2.5% dilution of different meat digests. For each digest, three replicates were performed; blank digests (digestive juices without meat) and untreated controls (30 µL DMEM added) were performed in duplicate. The impedance signal was analyzed by normalizing data of each well to the last measurement before treatment. $CI_{(normalized)} = CI_{(time\ x)}/CI_{(norm\ time)} \times 100$. The normalized cell indexes of three independent experiments were used for graphical result representation and cytotoxicity measurement.

Genotoxicity of meat digests using γ-H2AX assay

The effect of the different meat digests on DNA damage in the HT-29, HCT-116 and Caco-2 cell lines was assessed indirectly using immunofluorescence staining for γ-H2AX, a marker of DNA double strand break repair. Cells were seeded at a density of 5000 cells/well (Caco-2) or 10000 cells/well (HCT-116 and HT-29) in a 96-well plate. One day after plating, the cells were incubated for 24 h at a temperature of 37°C with a 2.5% dilution of the meat digests. Each treatment was performed in triplicate with an additional blank digest (digestive juices without meat) and untreated control, and the experiment was repeated at three different days.

Cells were fixed with 4% paraformaldehyde, washed with phosphate buffered saline (PBS) and permeabilized for 5 minutes with 0.5% Triton-X in PBS. After washing, cells were blocked with 50% fetal bovine serum in PBS (50% FBS/PBS) for 30 min and subsequently incubated for one hour with rabbit polyclonal antibodies against γ-H2AX (Abcam, ab2893) diluted 1:1000 in 50% FBS/PBS. After washing with PBS, goat anti-rabbit Alexa fluor 555 antibodies (Life Technologies) diluted 1:600 in 50% FBS/PBS were added for one hour. Afterwards, the cells were washed, stained with 5 ng/mL DAPI (Life Technologies) for 5 minutes and preserved in PBS at 4°C until image acquisition. For microscopy, a widefield microscope, type Nikon-Ti (Nikon Instruments, Paris, France) was used. Automated image acquisition was performed using a 20x (NA=0.5) dry objective for the entire well plate, and for each well a large image (2x2), with the inclusion of three z-slides (1 micrometer scale), was captured in the DAPI and TRITC channel. Focus was maintained across the well plate using the Nikon Perfect Focus System. For each condition the mean nuclear fluorescence intensity of γ-H2AX was measured using a dedicated image analysis script (InSCyDe.ijm) (De Vos et al., 2010) written for Fiji, image analysis freeware (fiji.sc).

Statistical analysis

Data on TBARS, 4-HNE and hexanal from the experiment on added reducing compounds were analyzed for each added compound and meat type separately, using a one-way ANOVA with the compound concentration as fixed factor. For the xCelligence cytotoxicity data, a two-way ANOVA was used for each cell line to study the influence of incubation with the different digests and incubation time on the outcome parameter (i.e. cell survival). Results obtained from the γ-H2AX assay were analysed using a mixed model, with treatment as fixed factor and the repetition day as random factor. Data on protein oxidation were analysed using a one-way

ANOVA with treatment as fixed factor. *Post hoc* comparisons were performed using the Tukey HSD test with $P < 0.05$ considered statistically significant.

RESULTS

Meat characteristics

Table 4.1 summarizes the characteristics of the beef model products used in the *in vitro* digestions. The fatty acid profile showed similar proportions of total SFAs, MUFAs and PUFAs in both beef products. However, the PUFA fraction of the high-fat beef product contained higher ALA and LA proportions and much lower LC *n*-3 and *n*-6 PUFA compared to the low-fat beef product. Haem-Fe and vitamin E concentrations were lower in the high-fat beef product compared to the low-fat beef product.

Table 4.1: Composition of the beef model products used in the *in vitro* digestion experiment.

	Unit	Low-fat beef			High-fat beef		
		mean	±	SD	mean	±	SD
Dry matter	g/100g	25.5	±	0.47	37.0	±	0.59
Protein	g/100g	22.4	±	0.15	19.8	±	0.02
Fat	g/100g	1.03	±	0.02	14.8	±	0.35
SFA	g/100g FAME	33.2	±	0.01	34.5	±	0.01
MUFA	g/100g FAME	38.5	±	0.45	40.1	±	0.17
PUFA	g/100g FAME	28.3	±	0.23	25.4	±	0.25
ALA	g/100g FAME	3.57	±	0.21	4.63	±	0.27
LC *n*-3 PUFA	g/100g FAME	5.78	±	0.19	0.311	±	0.00
LA	g/100g FAME	12.5	±	0.17	19.2	±	0.04
LC *n*-6 PUFA	g/100g FAME	4.47	±	0.05	0.487	±	0.04
Haem-Fe	mg/kg	20.9	±	0.22	15.8	±	0.16
Vitamin E	mg/kg	3.49	±	0.20	2.31	±	0.14

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; ALA = α-linolenic acid (C18:3 *n*-3); LC *n*-3 PUFA = long chain *n*-3 polyunsaturated fatty acids (C20:5 *n*-3; C22:5 *n*-3; C22-6 *n*-3); LA = linoleic acid (C18:2 *n*-6); LC *n*-6 PUFA = Long chain *n*-6 polyunsaturated fatty acids (C20:4 *n*-6; C22:4 *n*-6; C22:5 *n*-6); FAME = fatty acids methyl esters; SD = standard deviation.

LOP in digested low-fat and high-fat beef

Figure 4.1 shows a clear difference in LOP concentrations (P<0.001) between the digested low-fat and high-fat beef product without added reducing compounds (two digestion replicates on 8 different incubation days). Digestion of the high-fat beef product resulted in 11-fold higher TBARS, 14-fold higher 4-HNE and 8-fold higher hexanal concentrations, compared to the low-fat beef product.

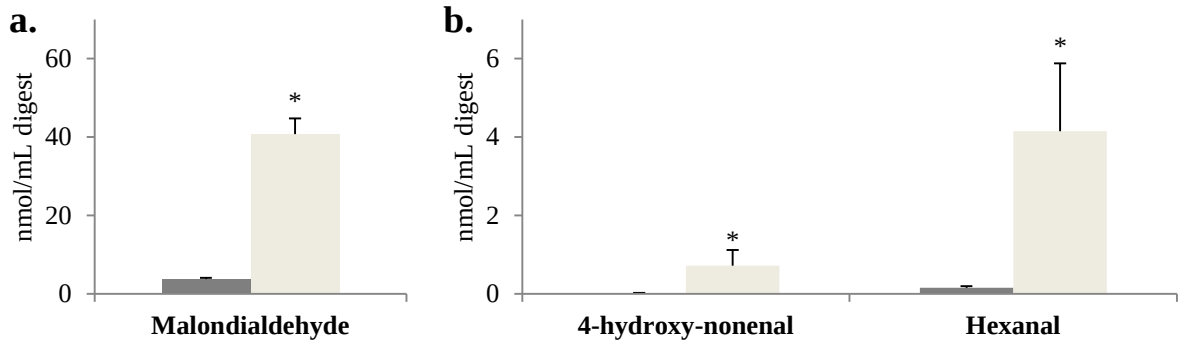

Figure 4.1: Lipid oxidation products after *in vitro* digestion of a ( ) low-fat (1% fat) and ( ) high-fat (15% fat) beef product without added compounds. Bars represent mean values (n=16). Error bars represent standard deviation. * refers to significant differences (P<0.05) between meat products.

Influence of reducing compounds on LOP formation during digestion

The effects of lipophilic and hydrophilic reducing compounds on TBARS, 4-HNE and hexanal concentrations during digestion of the low-fat and high-fat beef products, are shown in Figures 4.2 and 4.3, respectively. During digestion of the low-fat beef product, all hydrophilic compounds and quercetin significantly decreased LOP concentrations (all P<0.001), but not α-tocopherol and silibinin. However, addition of ascorbic acid, ferulic acid and caffeic acid to the low-fat beef digestion reduced LOP to a lesser extent when added at the highest doses. During the high-fat beef digestion, all lipophilic compounds clearly decreased LOP formation at all added doses (all P<0.02), with the exception of silibinin on TBARS formation. In contrast, a dose-dependent increase in 4-HNE formation was observed when the hydrophilic ascorbic acid or gallic acid were

added to the high-fat beef digestion (all P<0.01). Similarly, a dose-dependent increase in TBARS and hexanal formation was observed when ascorbic acid was added (all P<0.01), while gallic acid slightly decreased TBARS formation (P<0.001) and had no influence on hexanal formation. Addition of the other hydrophilic compounds resulted in higher 4-HNE formation (significant for ferulic acid and chlorogenic acid; trend (P=0.066) for caffeic acid) when added at the lowest dose, with a gradual decrease to no effect or antioxidant effect along with higher added doses. All phenolic acids reduced TBARS formation (P<0.001), albeit to a lesser extent for chlorogenic acid. Addition of doses up to 10 mg chlorogenic acid to the digestion of the high-fat beef caused an increase in hexanal formation, after which a decrease to the level of the control was observed at the highest dose. Addition of caffeic acid and ferulic acid resulted in a dose-dependent hexanal decrease (all P<0.005).

LOP in newly digested samples for cell line toxicity measurements

Compared to the previous digested samples, the new digests (n=3) for use in cyto- and genotoxicity assays contained similar amounts of LOP, as presented in Table 4.2.

Table 4.2: Lipid oxidation products in *in vitro* duodenal digests of the selected treatments for use in the cell lines.

	Unit	Low-fat beef		High-fat beef		High-fat beef + 5 mg AA		High-fat beef + 5 mg Qu	
MDA	nmol/mL	3.31	± 0.26	39.6	± 0.10	53.8	± 1.52	13.7	± 1.25
4-HNE	nmol/mL	0.03	± 0.00	0.36	± 0.04	0.55	± 0.04	0.02	± 0.01
HEX	nmol/mL	0.17	± 0.01	2.72	± 0.14	4.44	± 0.50	0.36	± 0.07

MDA = malondialdehyde; 4-HNE = 4-hydroxy-nonenal; HEX = hexanal; AA = ascorbic acid; Qu = quercetin.

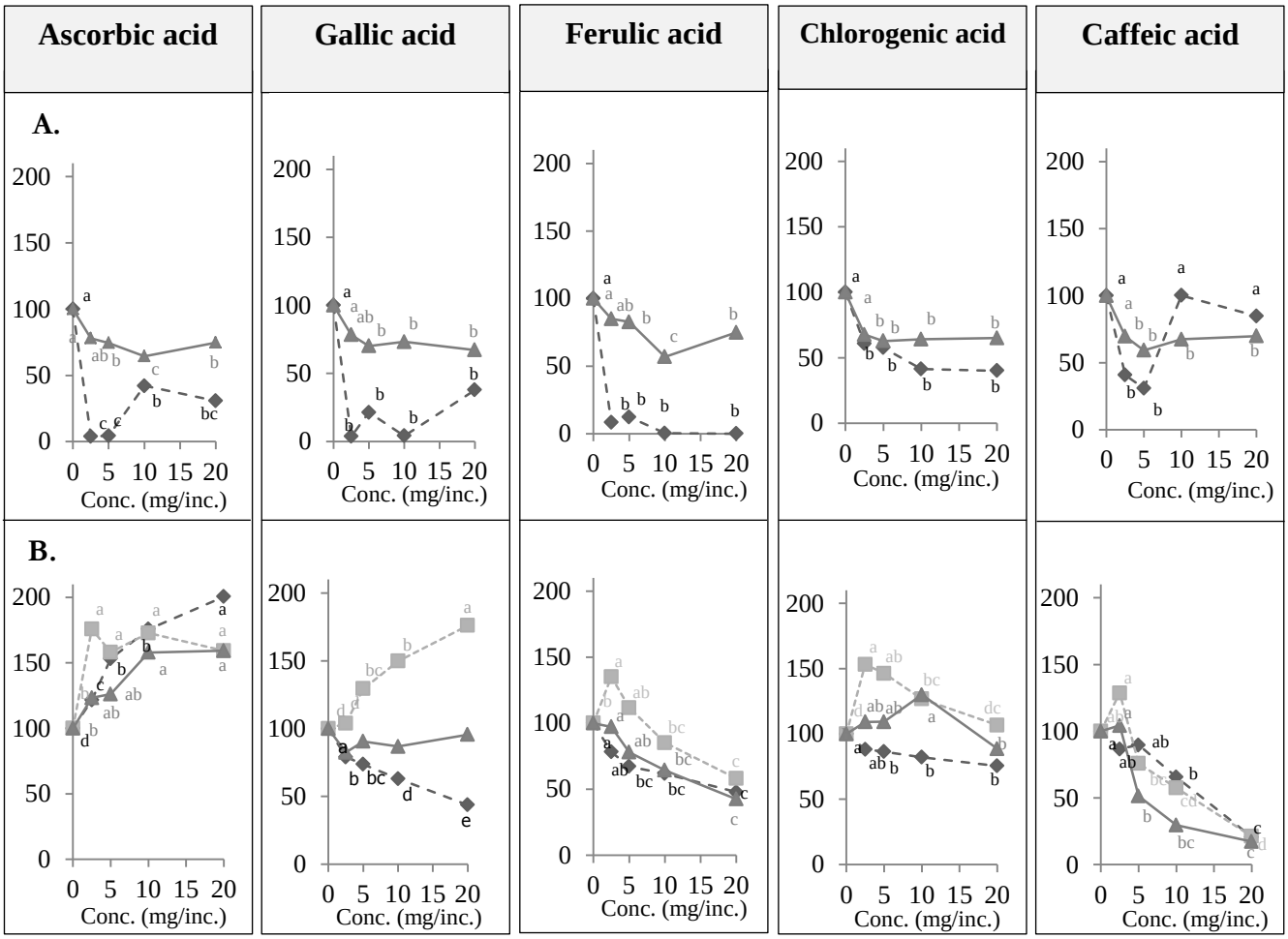

Figure 4.2: The effect of hydrophilic reducing compounds in different doses (0, 2.5, 5, 10, 20 mg/incubation) on malondialdehyde (---◆---), 4-hydroxy-nonenal (---■---) and hexanal (—▲—) concentrations (expressed relative (%) to control incubation without added compounds) in the digested low-fat (A. above) and high-fat (B. below) beef products. The effect of added compounds on 4-HNE concentrations during low-fat beef digestion was not reported as 4-HNE concentrations were close to the detection limit. Data labels refer to significant differences ($P<0.05$) among different concentrations.

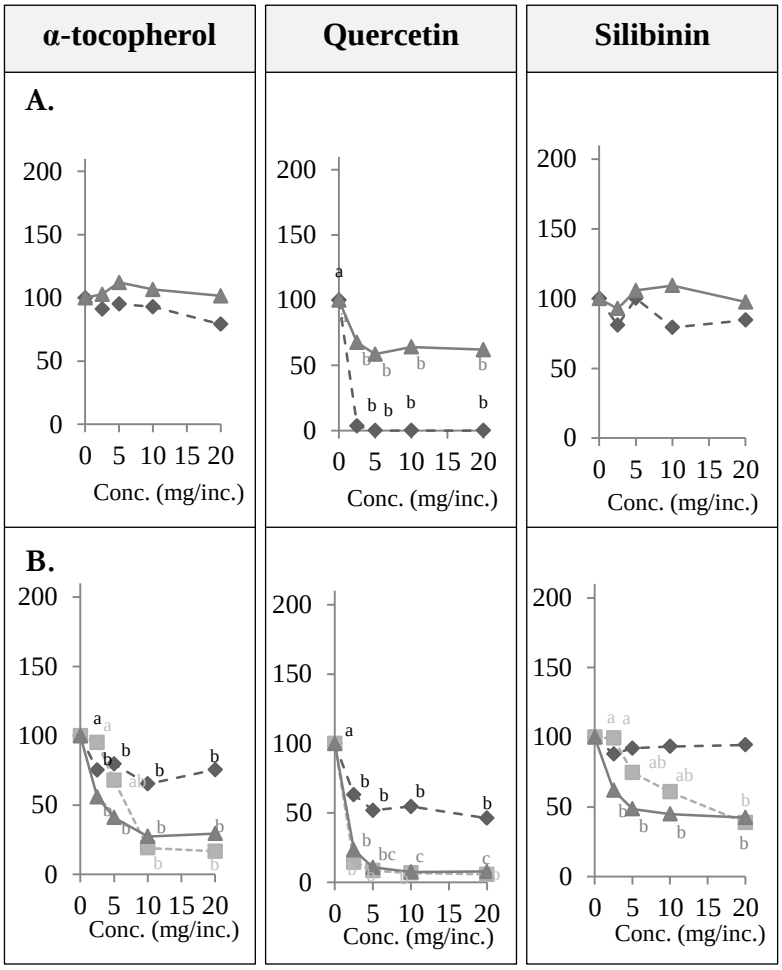

Figure 4.3: The effect of lipophilic reducing compounds in different doses (0, 2.5, 5, 10, 20 mg/incubation) on malondialdehyde (---◆---), 4-hydroxy-nonenal (---■---) and hexanal (—▲—) concentrations (expressed relative (%) to control incubation without added compounds) in the digested low-fat (A. above) and high-fat (B. below) beef products. The effect of added compounds on 4-HNE concentrations during low-fat beef digestion was not reported as 4-HNE concentrations were close to the detection limit. Data labels refer to significant differences ($P<0.05$) among different concentrations.

Real-time monitoring of the cytotoxicity of meat digests using xCelligence

Blank digests decreased cell survival (P<0.001) in comparison with control, untreated cells (Figure 4.4). Therefore, we corrected for this cytotoxicity by expressing survival after exposure to the meat digests relative to survival of the cells after exposure to the blank digests. The sensitivity of the three cell lines for the different digests varied significantly (P<0.001), with the Caco-2 cell line being more sensitive than the HT-29 and the HCT-116 cell lines, yet similar cytotoxic effects could be observed in all three cell lines. Incubation time highly influenced the corrected cell survival (P<0.001), with each time point (i.e. 1, 6, 12, 24, 48 and 72 hours) significantly different from one another, except for 48 h incubation *vs.* 72 h incubation (P=0.526).

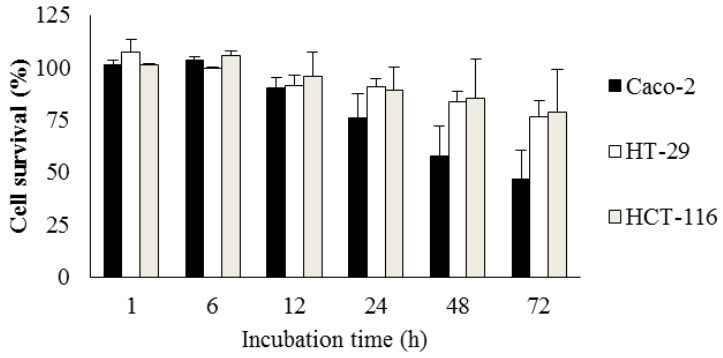

Figure 4.4: Time-dependent (1, 6, 12, 24, 48 and 72h incubation) cytotoxicity (xCelligence RTCA) profiles of human colorectal carcinoma cell lines (Caco-2, HT-29, HCT-116) after incubation with 2.5% blank digests (digestive juices without meat).

As shown in Figure 4.5, incubation with the low-fat beef digest clearly induced less cytotoxicity than incubation with the high-fat beef digest (P<0.001). No significant difference in cell survival after incubation with high-fat beef digests with or without 5 mg ascorbic acid could be observed. Digests of the high-fat beef product with 5 mg of quercetin induced significantly less cytotoxicity on the HCT-116 cell lines in comparison with incubation with the high-fat beef digests without quercetin (P=0.032). However, this effect was marginal, and absent in the other cell lines.

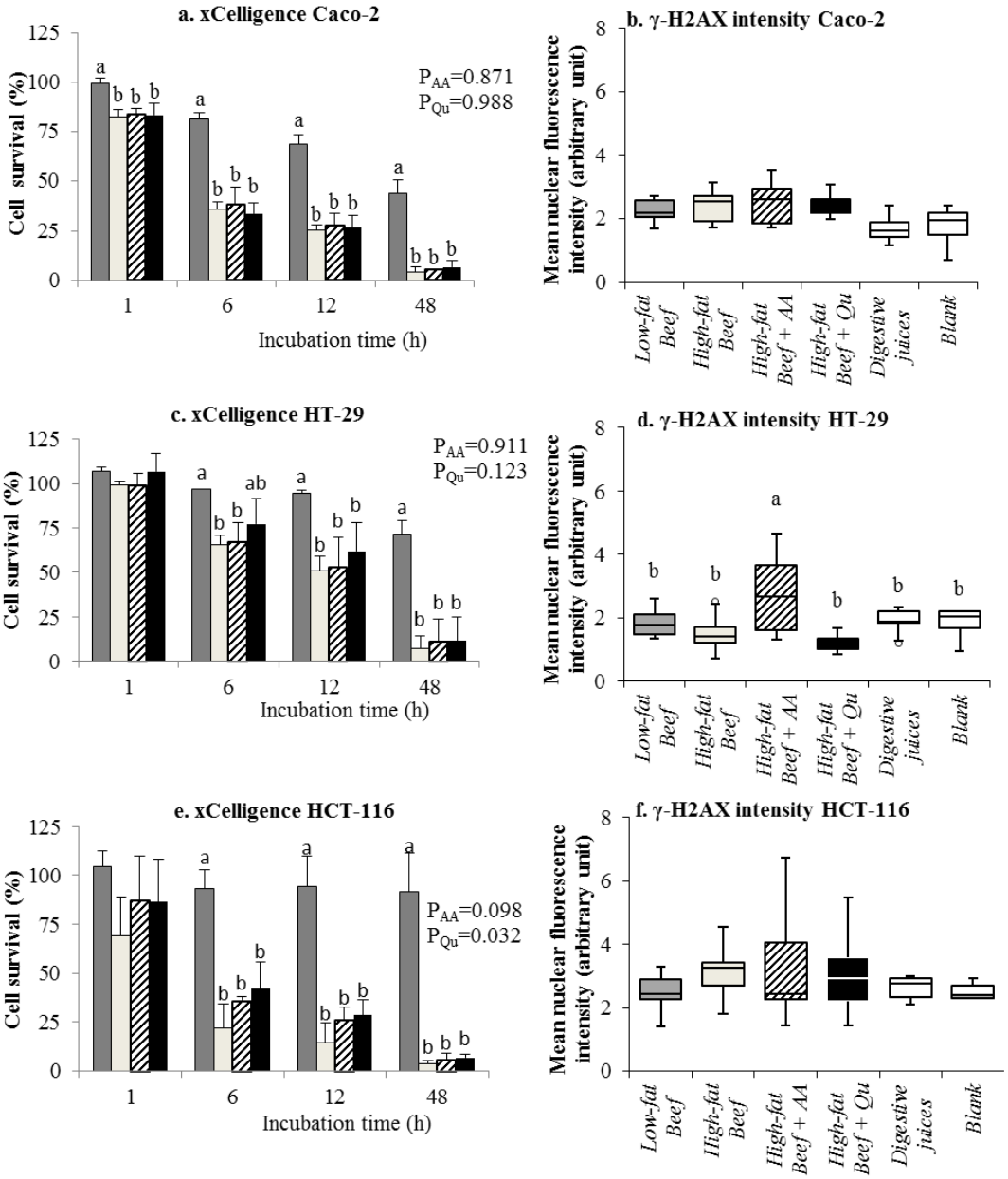

Figure 4.5: Time-dependent (1, 6, 12 and 48h incubation) cytotoxicity (xCelligence RTCA) and genotoxicity (24h incubation, γ-H2AX) profiles of human colorectal carcinoma cell lines (Caco-2, HT-29, HCT-116) after incubation with different meat digests (■ Low-fat beef; ▫ High-fat beef, ▨ High-fat beef + 5 mg ascorbic acid and ■ High-fat beef + 5 mg quercetin). Toxicity data were derived from normalized xCelligence plots. Cell survival was corrected for the toxic effect of blank incubation (digestive juices without meat) by setting survival after exposure to blank digests at 100%. P_{AA} and P_{Qu} represent the P-values of the effect of ascorbic acid and quercetin, respectively. The boxes in the genotoxicity graphs (γ-H2AX) represent the interquartile range (50% of the values) and the whiskers the total range (without outliers). The horizontal line within each box indicates the median metric value. Data labels indicate significant differences ($P<0.05$) among different treatments within the same time point. ** indicates significant difference ($P<0.01$) between the grouped meat digests *vs.* control treatments.

Genotoxicity of meat digests using γ-H2AX assay

DNA damage was measured by γ-H2AX fluorescence intensity after exposure of the three cell lines to the selected meat digests and blank samples (Figure 4.5). No significant differences were observed between the low-fat and high-fat beef digests in any cell line. High-fat beef digests with added ascorbic acid resulted in higher γ-H2AX intensity compared to all other treatments, however only in the HT-29 cell line (P=0.002).

Protein oxidation

The digests of the high-fat beef product contained nearly 3 times higher PCC concentrations compared to the low-fat beef digests (Figure 4.6). Addition of ascorbic acid did not alter PCC in the high-fat beef digests, while quercetin drastically reduced PCC concentrations to the level of the low-fat beef digests.

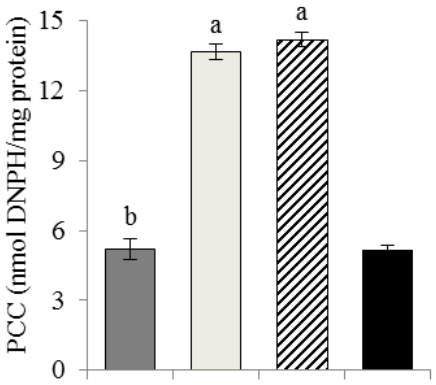

Figure 4.6: Protein oxidation measured by protein carbonyl compounds (PCC) in the selected digesta applied on the colorectal carcinoma cell lines. (■ Low-fat beef; ▒ High-fat beef, ▨ High-fat beef + 5 mg ascorbic acid and ■ High-fat beef + 5 mg quercetin). Data labels indicate significant differences (P<0.05).

DISCUSSION

The aim of this study was to elucidate whether (i) the addition of different concentrations of hydrophilic or lipophilic reducing compounds results in a decreased formation of LOP during the *in vitro* digestion of a low-fat and high-fat beef product, and whether (ii) a decreased LOP formation results in decreased cyto-/genotoxicity when human colorectal carcinoma cell lines are exposed to the digests. Sirota et al. (2013) showed that *in vitro* digestions were highly predictive for the *in vivo* effect of polyphenols on postprandial plasma MDA in humans. Our results clearly showed that the antioxidant or pro-oxidant activity of the tested compounds during digestion greatly depended on their concentration, their hydrophilic/lipophilic nature and the amount of fat in the meat products. Cell survival experiments confirmed that especially high-fat beef digests contain cytotoxic potential towards human colorectal carcinoma cells, which may contribute to initiation and/or progression of colorectal carcinogenesis.

The digestion of the high-fat beef product compared to the low-fat beef resulted in a 11-, 14- and 8-fold higher formation of TBARS, 4-HNE and hexanal respectively, corresponding to its higher fat content. In a previous experiment, we observed similar findings where addition of lard to pork model products increased LOP before and during digestion, however no differences in TBARS and hexanal were observed between digested pork products containing 5 and 20% fat, while this was the case for 4-HNE (Chapter II). In this previous study, the digested pork contained only 2.6 mg haem-Fe / kg meat, which is about 6 to 8 times lower than the beef used in this study. Therefore, the rather low haem-Fe levels in pork could have been the limiting factor during oxidation and explain the rather low difference in LOP concentrations between the digested 5 and 20% fat pork products.

During the digestion of the low-fat beef product, all hydrophilic compounds and quercetin exhibited clear antioxidant behavior, plausibly by scavenging ROS produced during the Fenton

reaction (Kohen & Nyska, 2002). It is unclear to us why quercetin exerted an antioxidant effect during the low-fat beef digestion in contrast to the other lipophilic reducing compounds, but it is likely that additional antioxidant characteristics apart from their polarity influence their effect. The lipophilic compounds exhibited efficient antioxidant behavior during the digestion of the high-fat beef product, while the hydrophilic compounds exerted an ambiguous pro- or antioxidant effect, depending on their concentration. A hypothesized reaction mechanism explaining the antioxidant or pro-oxidant outcome of the different added reducing compounds during the high-fat beef digestion is proposed in Figure 4.7. The hydrophilic compounds are able to reduce Fe^{3+} to Fe^{2+} (Moran et al., 1997) thereby stimulating the Fenton reaction and formation of ROS. During the digestion of the low-fat beef, the produced ROS are simultaneously scavenged by the added hydrophilic antioxidants. However, when a large lipid fraction is present, as is the case for the high-fat beef product, ROS may migrate into the lipid compartment where they cannot be reached by the hydrophilic antioxidants and hence stimulate the oxidation of *n*-3 and *n*-6 PUFAs.

The effect of caffeic acid, ferulic acid and chlorogenic acid on oxidation during digestion of the high-fat beef product was characterized by a 4-HNE peak formation at the lowest added dose, after which a gradual decrease was observed. In accordance, Kuffa et al. (2009) found that addition of low concentrations of grape seed extract during simulated gastric digestion of high-fat (30%) turkey meat had a pro-oxidant effect, while higher concentrations exerted an antioxidant effect. Schwarz et al. (1996) described ferulic acid and caffeic acid to be nearly 100% hydrophilic in a water/oil system at neutral pH but their partitioning in the water compartment decreased to respectively 77.3% and 90.4% when the pH of the system was dropped to 3, similar to stomach conditions. This partitioning effect was not observed for gallic acid, which remained nearly 100% hydrophilic in the acid water/oil system. As a result of the amphiphilic nature of caffeic acid and ferulic acid in acid heterophasic systems, it can be expected that these compounds both stimulate

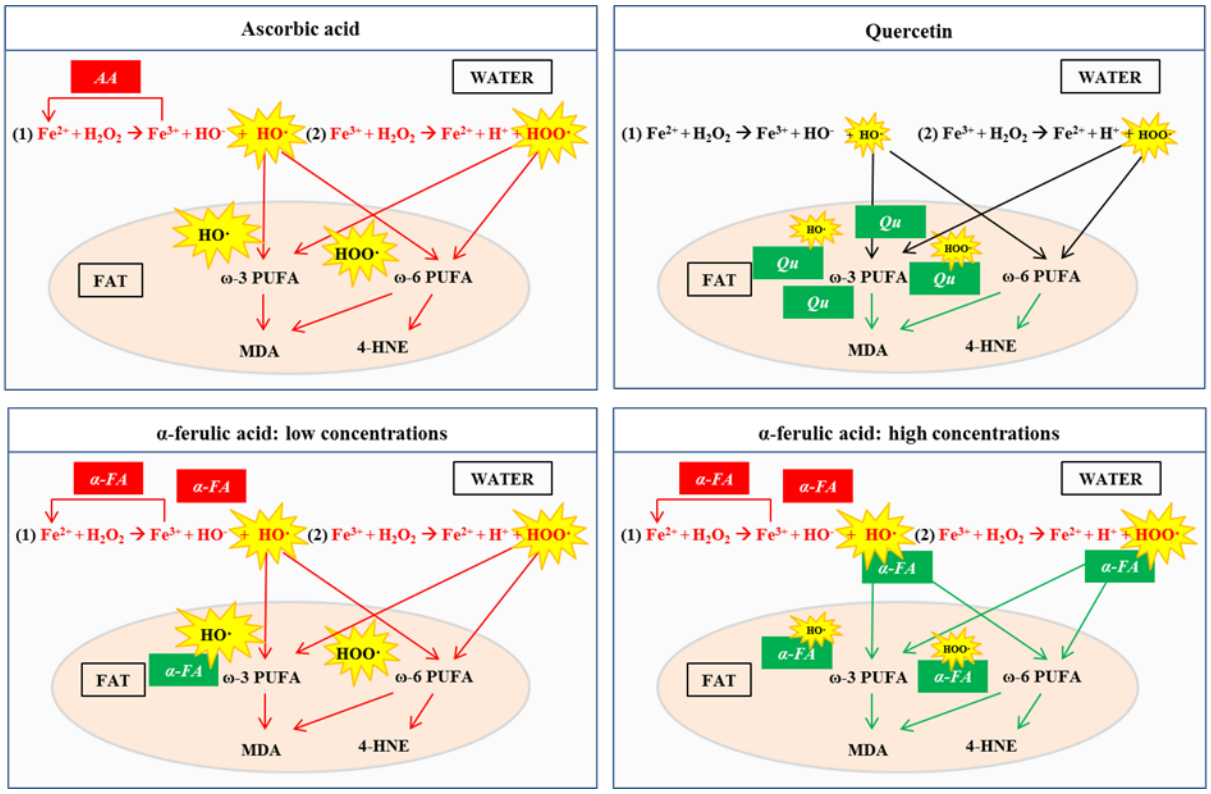

Figure 4.7: Proposed underlying mechanism explaining the anti- or pro-oxidant effect of tested compounds during the digestion of the heated high-fat beef product in the acid stomach. Upper Left: water soluble ascorbic acid (AA) reduces Fe^{3+} to Fe^{2+} whereby the Fenton reaction is maintained to produce hydroxyl radicals (HO·) and hydroperoxyl radicals (HOO·). Produced radicals can escape into the lipid compartment where they are unreachable by ascorbic acid and hence stimulate the formation of malondialdehyde (MDA) and 4-hydroxy-nonenal (4-HNE) (cf. gallic acid). Upper Right: quercetin (Qu) partitions in the fat compartment. When radicals formed during the Fenton reaction reach the fat compartment, quercetin can easily scavenge them before they cause damage to the PUFAs and therefore prevent MDA and 4-HNE formation (cf. α-tocopherol, silibinin). Lower Left: during the acid stomach digestion, ferulic acid (FA) will mainly partition in the water compartment, with a small proportion in the fat compartment. At low concentrations of FA, the Fenton stimulating effect of FA will dominate the radical scavenging ability of FA in the fat compartment whereby the net effect is increased oxidation (cf. caffeic- and ferulic acid). Lower Right: at high concentrations of FA, the radical scavenging ability of FA in the fat compartment will be sufficient to compensate for the Fenton stimulating effect in the water compartment, resulting in a net antioxidant effect (cf. caffeic- and ferulic acid). Red arrows and frames indicate stimulation of oxidation while green arrows and frames indicate inhibition of oxidation.

ROS formation in the water compartment by reducing Fe^{3+} to Fe^{2+} (Moran et al., 1997) and inhibit lipid oxidation by scavenging ROS in the lipid compartment. Furthermore, as observed in our results, higher doses of caffeic acid and ferulic acid may decrease lipid oxidation compared to the lowest dose, since higher concentrations would partition in the lipid compartment and higher concentrations in the water phase could not only reduce Fe^{3+} to Fe^{2+}, but also scavenge produced ROS. It is however not clear why this peak formation was not observed for hexanal (except for chlorogenic acid) and for TBARS during the high-fat beef digestion.

It should also be kept in mind that lipid oxidation is a chain reaction and addition of lard during meat processing and cooking stimulates this reaction in meat and during its digestion (Chapter II and III) Therefore, the reducing compounds added to the low-fat and high-fat beef product were likely exposed to an environment at a different phase of the oxidative chain reaction. *In vitro* oxidation systems previously showed that the pro- or antioxidant effect of added hydrophilic reducing compounds depended on their concentration and if they were added during the initiation or propagation phase of oxidation (Bagnati et al., 1999; Yamanaka et al., 1997). Considering this, adding hydrophilic antioxidants before heating may result in a different outcome than the effects reported here, since heating of meat drastically increases oxidative reactions during meat processing and digestion (Chapter III). More research is needed to confirm these hypotheses.

We also aimed to elucidate whether decreased LOP formation in the digests resulted in lower cyto- and genotoxicity when applied to different colorectal cancer cell lines. These cell lines typically have a mutation in the Apc gene (a tumor suppressor gene) and/or the β-catenin gene (a proto-oncogene). We have chosen to investigate the selected digests on various cell lines with a different genetic background in terms of Apc and β-catenin mutation status to avoid cell-type specific effects; Caco-2 (mutated Apc and mutated β-catenin), HT-29 (mutated Apc and wildtype

β-catenin) and HCT-116 (wildtype Apc and mutated β-catenin) (El-Bahrawy et al., 2004). The applied digests exerted similar cytotoxic effects in the different cell lines, with the Caco-2 cell line being more sensitive. Previous research demonstrated cytotoxic effects of hemoglobin and hemin, and not of inorganic iron, on HT-29 cells (Glei et al., 2006; Klenow et al., 2009). Absorption and metabolic degradation of haem-Fe is considered to increase the production of ROS in the cell, which in turn may cause cellular toxicity (Ishikawa et al., 2010). In the present experiment, we exposed the cell lines to selected beef digests containing different amounts of LOP; the low-fat beef digests (low in LOP formation), high-fat beef digests (high in LOP formation), high-fat beef digest with ascorbic acid (stimulation of LOP formation) and high-fat beef digest with quercetin (inhibition of LOP formation). Especially the high-fat beef digests exerted a clear cytotoxic effect on the three cell lines, whereas this effect was far less pronounced for the low-fat beef digests. In a previous *in vivo* study, no effect was observed on fecal water cytotoxicity when rats were fed diets containing different amounts of fat. However, when haem-Fe was included in the diet, the fecal water of rats on the medium- and high-fat diets had a clear cytotoxic effect, while induction of cytotoxicity was much less pronounced in the low-fat diet (Sesink et al., 2000), in line with our results. This important interaction between haem-Fe and fat may explain why the relative risk for CRC is higher for processed meats than for fresh red meat. Indeed, processed meats do generally have a much higher fat content than fresh meat. On the other hand, the low-fat beef had a 1.13 fold higher protein content compared to the high-fat beef. It cannot be excluded that a higher amount of antioxidant peptides is released during digestion of the low-fat beef, which may interfere in the observed effects (Lafarga et al., 2014; Wang et al., 2015).

Haem-Fe ingested with fat from meat or other foods can generate lipid peroxyl radicals, which in turn induce DNA single strand breaks (Sawa et al., 1998). The measurement of phosphorylated histone H2AX (γ-H2AX) is a widely used assay to determine the cellular response to DNA

double strand breaks (Kuo et al., 2008). No significant difference in γ-H2AX intensity could be demonstrated in either cell line between the low-fat or high-fat beef digests (without added reducing compounds), whereas their concentration of LOP was clearly different. Possibly, oxidative stress mostly causes single strand breaks and base oxidation products instead of double strand breaks. Recently, Bastide et al. (2015) reported a clear phosphorylation of H2AX when normal Apc+/+ cells were exposed to 4-HNE, whereas this effect was negligible in premalignant Apc-/+ cells. Therefore, it would be interesting to investigate the effect of the digests on non-malignant colon cell lines in future studies.

Although the addition of quercetin drastically decreased LOP concentrations during the high-fat beef digestion, close to the level of LOP in the low-fat beef digests, the decreased cytotoxicity was negligible, as was the effect on genotoxicity. It has been reported that in rat lung epithelial cells, quercetin exerted efficient antioxidant behavior by preventing H_2O_2-induced DNA damage (Boots et al., 2007). On the other hand, the oxidized products of quercetin simultaneously exerted toxic effects by reducing GSH, increasing LDH leakage and increasing cytosolic free calcium concentrations (Boots et al., 2007). This so-called "quercetin paradox" could possibly be responsible for the only marginal protective effect observed in our cell lines. Furthermore, in an epidemiological case-control study (1163 cases *vs.* 1501 controls), a high quercetin intake was associated with lower proximal colon cancer risk only when accompanied with a high consumption of fruits. When fruit intake was low, a high quercetin intake was associated with an increased distal colon cancer risk (Djuric et al., 2012). Therefore, more research is warranted before application of quercetin into meat processing.

Addition of ascorbic acid during high-fat beef digestion resulted in clearly higher LOP formation. This was not accompanied with altered cytotoxicity, but an 85% increase in γ-H2AX intensity

was observed in the HT-29 cell line, but not in other cell lines. However, in all cases there was a large variability in the cell response as evidenced by the span of the box plots.

Since the LOP concentrations could not explain the cyto- and genotoxic effects of the digests, measuring the PCC in the selected digesta could contribute to explaining these effects on the cell lines. Protein oxidation matched the results of the LOP in the digesta, confirming the oxidative status of the applied digesta. Likely, other meat-related compounds such as NOCs and HCAs (Demeyer et al., 2015) also contribute to the toxicity of the digests. Since microbiota are described to have a role in CRC development (Sears et al., 2014), it is also expected that fermentation of the digests by the intestinal microbiota might reveal different results *in vivo*. More research is warranted to explain the cyto- and genotoxic potential of meat digests.

In conclusion, our results suggest that antioxidants partitioning in the aqueous phase during the digestion of oxidized high-fat beef products, display a pro-oxidant activity when added after heating of meat. Since processed meat usually contains a considerable amount of fat, future digestion studies on antioxidant activity should also consider the presence of a large fat compartment. Oxidation processes during digestion are determined by a complex interplay between the "polar paradox theory" (Shahidi and Zhong, 2011), the extent of initial oxidation and composition of the meat before digestion, and the concentration and nature of the added antioxidant. Studying the partitioning behavior of antioxidants in acid heterophasic systems, such as present in the stomach, would improve our insight on their mode of action. In addition, lipid oxidation was only assessed in samples at the end of small intestinal digestion in the present study. Quantification of LOP in the separate segments of the alimentary tract could result in a better understanding on how reducing compounds affect LOP formation during digestion. Furthermore, since we observed a clear cytotoxic effect of high-fat beef digests compared to low-

fat beef digests, the fat in haem-Fe rich processed meat products might be a contributing or mediating factor for its negative impact on human health. Since the cyto- and genotoxic potential of the digests applied on the CRC cell lines could not be explained by the presence of LOP, more research is warranted to pinpoint the underlying mechanisms.

Chapter V

Short-term beef consumption promotes systemic oxidative stress, TMAO formation and inflammation in rats, and dietary fat modulates these effects

Adapted from:

Van Hecke, T., Jakobsen, L.M., Vossen, E., Guéraud, F., De Vos, F., Pierre, F., Bertram, H.C., De Smet, S. Short-term beef consumption promotes systemic oxidative stress, TMAO formation and inflammation in rats, and dietary fat modulates these effects. *Food and Function*, submitted.

ABSTRACT

A high consumption of red (processed) meat is associated with a higher risk to develop several chronic diseases in which oxidative stress, trimethylamine-*N*-oxide (TMAO) and/or inflammation are involved. We aimed to elucidate the effect of white (chicken) *vs.* red (beef) meat consumption in a low *vs.* high dietary fat context (2×2 factorial design) on oxidative stress, TMAO and inflammation in Sprague-Dawley rats. Higher TBARS concentrations were found in gastrointestinal contents (up to 96% higher) and colonic tissues (+8.8%) of rats fed the beef diets (all $P<0.05$). The lean beef diet resulted in lower blood glutathione, higher urinary excretion of the major 4-HNE metabolite, and higher plasma C-reactive protein, compared to the other dietary treatments (all $P<0.05$). Rats on the fat beef diet had higher renal TBARS (+24.4% compared to all other diets) and heart TBARS (+12.9% compared to lean chicken) and lower liver vitamin E (-26.2% compared to lean chicken) (all $P<0.05$). Rats on the fat diets had lower plasma vitamin E (-23.8%), lower brain TBARS (-6.8%) and higher plasma superoxide dismutase activity (+38.6%), higher blood glutathione (+16.9%) (all $P<0.05$) and tendency to higher ventral prostate TBARS (+14.5%, $P=0.078$) and prostate weight (+18.9%, $P=0.073$). Consumption of beef diets resulted in higher urinary trimethylamine (4.5-fold) and TMAO (3.7-fold) concentrations ($P<0.001$). In conclusion, consumption of a high beef diet may stimulate gastrointestinal and/or systemic oxidative stress, TMAO formation and inflammation, depending on the dietary fat.

INTRODUCTION

Haem-Fe and fat, abundantly present in processed red meat, give rise to the formation of toxic LOP such as TBARS and 4-HNE during meat processing and digestion (Pierre et al.,

2004; Chapters I and II). Following red meat consumption, MDA concentrations increase in plasma (Gorelik et al., 2008b; Toden et al., 2010) but data are lacking on its impact on systemic oxidative stress parameters. In large-scale epidemiologic studies, a high red (processed) meat consumption is positively associated with an increased risk to develop a range of chronic diseases including CRC, coronary heart disease and diabetes (Chan et al, 2011; Micha et al., 2010; 2012). These are all diseases in which oxidative stress is believed to exert a role in their etiology and/or progression (Corpet, 2011; Rains & Jain, 2011; Chen & Keaney, 2012).

Next to oxidative stress, inflammation contributes to the pathogenesis of these diseases. Low-grade inflammation is associated with an increased risk to develop CRC and breast cancer (Il'yasova et al., 2005), coronary heart disease (IL6R Genetics Consortium Emerging Risk Factors Collaboration, 2012) and diabetes (Pradhan et al., 2001). Moreover, epidemiologic studies show that a high red meat diet is associated with increased C-reactive protein (CRP) (Azadbakht & Esmaillzadeh, 2009; Montonen et al., 2013; Ley et al., 2014), a common marker to detect inflammation. However, controlled dietary studies that investigated the latter association are scarce (Hodgson et al., 2007; Guéraud et al., 2015).

The colonic fermentation of *L*-carnitine, abundant in red meat, to trimethylamine (TMA), which is later converted to trimethylamine-*N*-oxide (TMAO), was recently suggested to increase the risk to develop CVD by its proatherogenic properties (Koeth et al., 2013). Chronic dietary exposure to TMAO also induced functional impairment of the kidneys in mice (Tang et al., 2015). However, the possible involvement of TMAO has also received criticism (Johri et al., 2014) since supplemental *L*-carnitine actually improves some features of CVD, and consumption of fish, which is associated with lower CVD risk, dramatically

increases TMAO. Recently, plasma TMAO was even reported to be correlated with reduced aortic lesions in mice (Collins et al, 2016).

In this study, we wanted to elucidate the individual and combined effects of haem-Fe and fat content in meat products on the systemic redox and inflammation status, and TMAO formation during a short-term rat feeding experiment. Therefore, meat products were produced based on chicken and beef protein, which are low and high sources of haem-Fe (Lombardi-Boccia et al., 2002) respectively, and were either or not mixed with lard, representing high fat meat products. The oxidative status of the rats was evaluated by measurement of TBARS in organs and excreta, the major urinary 4-HNE metabolite 1,4-dihydroxynonane mercapturic acid (DHN-MA) (Guéraud et al, 2006), oxidative modification of low-density-lipoproteins (ox-LDL) and effects on various antioxidant defense mechanisms such as vitamin E, glutathione (GSH), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD). Urinary TMA and TMAO were quantified using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Quantification of CRP was used in this study as a marker of low-grade inflammation.

MATERIALS AND METHODS

Manufacturing of meat samples and animal diets

Lean meat samples from the *m. pectoralis profundus* of chicken and beef were purchased and manually chopped. Next to the lean chicken and lean beef products, a fat chicken and a fat beef product were made by adding lard to the lean muscles at a proportion of 20% on the final product. The meat samples were minced in a grinder (Omega T-12) equipped with a 10 mm

plate, followed by grinding through a 3.5 mm plate, vacuum packing in plastic bags, and heating for 70 min at 70°C in a warm water bath. After manufacturing, all meat samples were homogenized using a food processor. Subsequently, 4 meat diets were formulated to contain 65% of the lean meat or fat meat product (w/w) (Table 5.1). Finally, the experimental diets were vacuum-packed in daily portions (± 70 g) and stored at -20°C.

Table 5.1: Ingredients and composition of the experimental diets.

		Chicken		Beef	
	Unit	**Lean**	**Fat**	**Lean**	**Fat**
Ingredients					
Meat product	g/kg	650	650	650	650
- chicken muscle	*g/kg*	*650*	*550*	-	-
- beef muscle	*g/kg*	-	-	*650*	*550*
- Lard	*g/kg*	-	*100*	-	*100*
Sucrose	g/kg	200	200	200	200
Corn starch	g/kg	79.2	79.2	79.2	79.2
Cellulose	g/kg	22	22	22	22
Safflower oil	g/kg	20	20	20	20
Mineral mix[a]	g/kg	20	20	20	20
Vitamin mix[b]	g/kg	6.3	6.3	6.3	6.3
Calcium phosphate	g/kg	1.3	1.3	1.3	1.3
Choline bitartrate	g/kg	1.2	1.2	1.2	1.2
Analyzed composition					
Dry matter	%	51.5	58.5	51.2	59.1
Crude protein	%	14.0	12.0	14.4	12.8
Crude fat	%	2.8	12.5	1.7	14.7
SFA	*% FA*	*16.9*	*32.4*	*18.4*	*33.5*
MUFA	*% FA*	*24.9*	*40.2*	*22.4*	*39.6*
PUFA	*% FA*	*58.2*	*27.3*	*59.3*	*26.9*
LA	*% FA*	*55.5*	*24.5*	*55.7*	*24.1*
LC n-6 PUFA	*% FA*	*1.6*	*1.3*	*1.8*	*1.2*
ALA	*% FA*	*0.4*	*0.4*	*1.2*	*0.5*
LC n-3 PUFA	*% FA*	*0.7*	*1.1*	*0.5*	*1.0*
DHA	*% FA*	*0.08*	*0.04*	*0.10*	*0.04*
Crude ash	%	2.6	2.3	2.4	2.2
TBARS	nmol/g	43.6	45.5	71.8	79.8
Vitamin E	mg/kg	10.9	11.8	8.0	9.9
Haem-Fe	mg/kg	*0.5*	*0.4*	*7.2*	*6.1*

(a) a modified Ca-P deficient mineral mix was used (TD.79055, Harlan laboratories); (b) AIN76 vitamin mix (MP Biomedicals); SFA=saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA=polyunsaturated fatty acids; LC *n*-3 PUFA=long chain *n*-3 polyunsaturated fatty acids (C20:5,*n*-3; C22:5,*n*-3; C22-6,*n*-3); DHA= Docosahexaenoic acid; ALA=α-linolenic acid (C18:3,*n*-3); LA=linoleic acid (C18:2,*n*-6); LC *n*-6 PUFA = Long chain *n*-6 polyunsaturated fatty acids (C20:4,*n*-6; C22:4,*n*-6; C22:5,*n*-6); FA = fatty acid

Chemical composition of the experimental diets

See Chapter I (p. 25).

Rat experiment and sample preparation

The rat experiment was conducted following the principles of laboratory animal care and the Belgian law on the protection of animals. The experimental protocol was approved by the Ghent University Ethical Committee (ECD 14/58). Twenty four male Sprague-Dawley rats (± 150 g) (Janvier laboratories, France) were given an adaptation period of 10 days and were housed per group of four rats. A standard laboratory diet (Ssniff R/M-N pellets) (Ssniff, Soest, Germany) and water were provided *ad libitum*. At the start of the experimental diets, rats were housed individually. The diets were offered *ad libitum* and refreshed daily. Body weight and daily food intake was monitored every 2 days. Following 10 days on the meat diets, the rats were housed 24h in a metabolic cage during which urine and feces were collected. Urine was collected in a container with 0.1 mL of 1% sodium azide (w/v) and was cooled by ice during the whole procedure. After 14 days on the meat diets, rats were anesthetized by 5% isoflurane gas and blood was collected from the abdominal aorta into heparin tubes until death occurred. Plasma was obtained by low speed centrifugation and immediately stored at -80°C. Subsequently, organs (eyes, heart, lungs, liver, pancreas, spleen, duodenum, colon, kidney, prostate, testis, brain) were removed, carefully rinsed with 0.9% NaCl solution, where after 1% triton-x-100 phosphate buffer (pH 7; 50 mM) was added to the organs in a 1/10 ratio (w/v). Subsequently, solutions were homogenized with an ultraturrax and centrifuged (15 min, 15.000 g, 4°C), after which the supernatant was filtered through glass wool and stored at -80°C in different aliquots. Untreated livers and stomach- and colonic contents were stored as such at -80°C.

Oxidative stress parameters

TBARS concentrations were measured colorimetrically according to a modified method of Grotto et al. (2007). TBARS were formed from the reaction of MDA with 2-thiobarbituric acid in an acid environment. After extraction in 1-butanol, the absorbance of the colored complex was measured colorimetrically at 532 nm. A standard curve with 1,1,3,3-tetramethoxypropane was used. Oxidized low-density-lipoproteins (ox-LDL) in plasma were quantified using a commercial ELISA kit (Cusabio Biotech, Wuhan, China). α-tocopherol was measured in plasma and untreated liver by reverse phased HPLC (Claeys et al., 2016). The concentrations of GSH and GSSG in the red blood cell fraction were determined by HPLC (Degroote et al, 2012). The oxygen radical absorbance capacity (ORAC) of plasma samples was measured fluorimetrically (Ou et al., 2001). The activity of gluthatione peroxidase (GSH-Px) in plasma and organ extracts was determined by measuring the oxidation of NADPH (Hernández et al., 2004) whereby one unit of GSH-Px activity was defined as the amount of extract needed to oxidize 1 μmol of NADPH per min at 25 °C. Superoxide dismutase (SOD) activity in plasma was determined using a commercial enzyme kit (Randox Laboratories, Crumlin, UK).

Inflammation

Quantification of C-reactive protein (CRP) in plasma was performed using a commercial ELISA kit (RayBiotech, Norcross, USA).

Urinary parameters

The urinary metabolite of 4-HNE, 1,4-dihydroxynonane mercapturic acid (DHN-MA) was quantified by competitive enzyme immunoassay as previously described by Guéraud et al. (2006). Uric acid and allantoin were measured by HPLC according to Lima et al. (2011).

Quantification of urinary TMA and TMAO using ^{1}H NMR spectroscopy

One mL urine was centrifuged (10 000 g, 4°C, 5 min) and 0.75 mL of the supernatant was mixed with 0.15 mL phosphate buffer (0.5M, pH 7.4) containing D_2O and 3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionic acid (TSP), left for a few minutes to allow precipitation of salt, then centrifuged (10 000g, 4°C, 5 min). A volume of 0.6 mL supernatant was transferred to an NMR glass tube. ^{1}H NMR spectra were recorded using a standard 90° pulse sequence (zgpr, Bruker BioSpin, Gmbh, Rheinstetten, Germany) with presaturation of the water resonance. The pulse length was 7.9 μs, and the recycle delay was 5 s. A total of 64 FIDs were acquired and acquisition parameters included 32K complex data points, a spectral width of 7289 Hz (12.15 ppm) and an acquisition time of 2.25s. Integrals were calculated in the TopspinTM 3.0 software (Bruker BioSpin, Gmbh, Rheinstetten, Germany). The regions used for quantification were as follows: TMA: 2.879-2.920 ppm, TMAO: 3.270-3.280 ppm and TSP: -0.009-0.009 ppm. The concentrations of TMA and TMAO were calculated according to the formula;

$$c(metabolite) = \frac{int(metabolite) * protons(metabolite)}{int(TSP) * protons(TSP)} \cdot c(TSP) \cdot \frac{vol(NMRtube)}{vol(urine)}$$

where 'int' is the integral, 'vol' is volume, and the concentration of TSP is 0.01147 mmol/L. The concentrations were expressed as 24 h excretion.

Statistics

A mixed model ANOVA procedure (SAS Enterprise Guide 7) was used with the fixed effects of species, fat content and species × fat content and the random effect of euthanization day. Tukey-adjusted post hoc tests were performed for all pairwise comparisons with $P<0.05$ considered significant.

RESULTS

Composition and characterization of the experimental diets

As expected, the fat diets had a higher dry matter, lower protein and higher fat content (Table 5.1). Fatty acid profiles were very similar for diets of the same fat content group. Compared to the fat diets, the lean diets had lower proportions of SFAs and MUFAs, while proportions of PUFAs were higher. The latter fraction mainly consisted of linoleic acid (LA). The beef diets contained more TBARS compared to the chicken diets, irrespective of the dietary fat content. The fat diets contained more vitamin E than the lean diets, whereas the beef diets contained less vitamin E than the chicken diets. Haem-Fe concentrations were approximately 15-fold higher in the beef diets compared to the chicken diets, with highest levels in the lean beef diets.

Animals

The initial and final body weight of rats on the different dietary treatments did not differ (Figure 5.1.A). Rats on the fat diets had a significantly lower feed intake (-25%) compared to rats on the lean diets (Figure 5.1.B), but there was no difference in metabolizable energy intake (P=0.751).

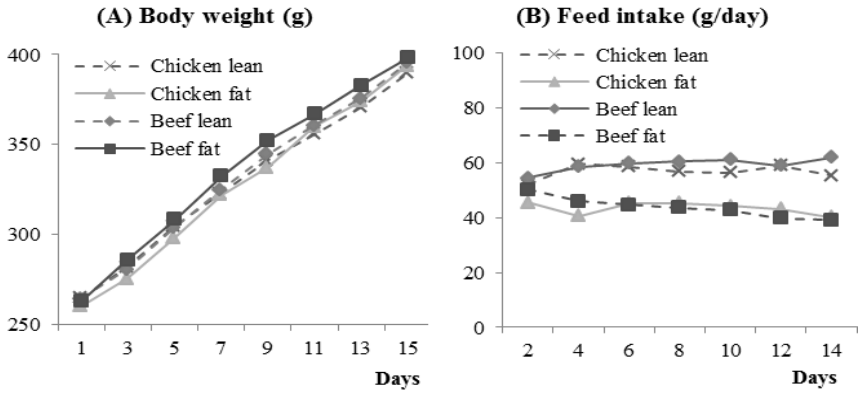

Figure 5.1: Body weight (A) and feed intake (B) during the experimental feeding period.

Animals on the fat diets had a significantly lower urine production (-31%), lower kidney weight (-9.1%) and tended to have higher weights of the ventral prostate lobe (+18.9%, P=0.073) compared to rats on the lean diets. Rats on the lean beef diet had significantly lower spleen weights compared to rats on the lean chicken diet (-18.8%).

Malondialdehyde throughout digestion

On dry matter base, approximately 2-fold higher TBARS concentrations were observed in the stomach content of rats on the beef diets and the fat chicken diet, compared to TBARS concentrations in undigested diets and colonic content (Figure 5.2). Rats on the lean chicken diet had 40% higher TBARS values in the stomach content compared to the diet before digestion. Digests of beef had significantly higher TBARS concentrations in all digestion phases.

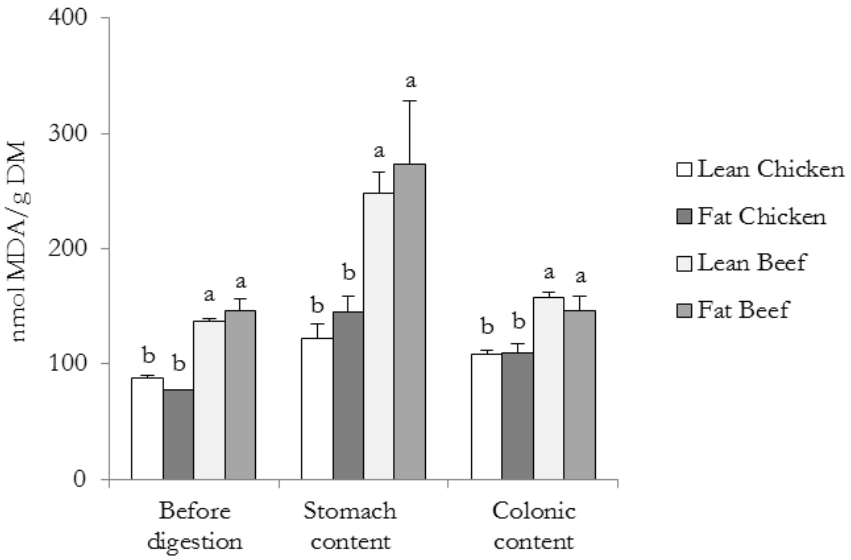

Figure 5.2: thiobarbituric acid reactive substances (TBARS) concentrations in diets before digestion, during stomach passage (total n=24) and colonic passage (total n=24), expressed in nmol TBARS per gram dry matter (DM). Different superscripts indicate significant differences (P<0.05) among diets in the same digestion stage.

Oxidative stress parameters in blood

Figure 5.3 shows the blood oxidative stress parameters. Rats on the fat diets had lower α-tocopherol concentrations in plasma (-23.8%), whereas they had higher SOD activity (+38.6%) and higher GSH concentrations (+16.9%) (all $P<0.05$) compared to rats on the lean meat diets. Furthermore, rats fed the lean beef diets had less GSH compared to rats on the fat chicken diet (-35.3 %) and fat beef diet (-22.8%) ($P<0.05$), and a trend ($P=0.061$) to less GSH compared to rats on the lean chicken diet (-20.8%). Furthermore, GSSG concentrations were significantly lower in plasma of rats on the lean beef diet compared to the lean and fat chicken diet. The GSSG:GSH ratio was significantly lower in rats on the beef diets (-22.6%) compared to rats on the chicken diets, with a trend ($P=0.064$) for a lower GSSG:GSH ratio in the lean beef group (-41.7%) compared to the lean chicken group. Rats fed the beef diets tended to have slightly higher ORAC values in plasma (+4.5%) compared to rats on the chicken diets. No significant differences were found in ox-LDL between the different diet groups, but the interaction term between species and fat content was significant, with lean chicken and fat beef diets showing non-significantly higher values. TBARS concentrations and GSH-Px activity in plasma were not significantly different among dietary treatments.

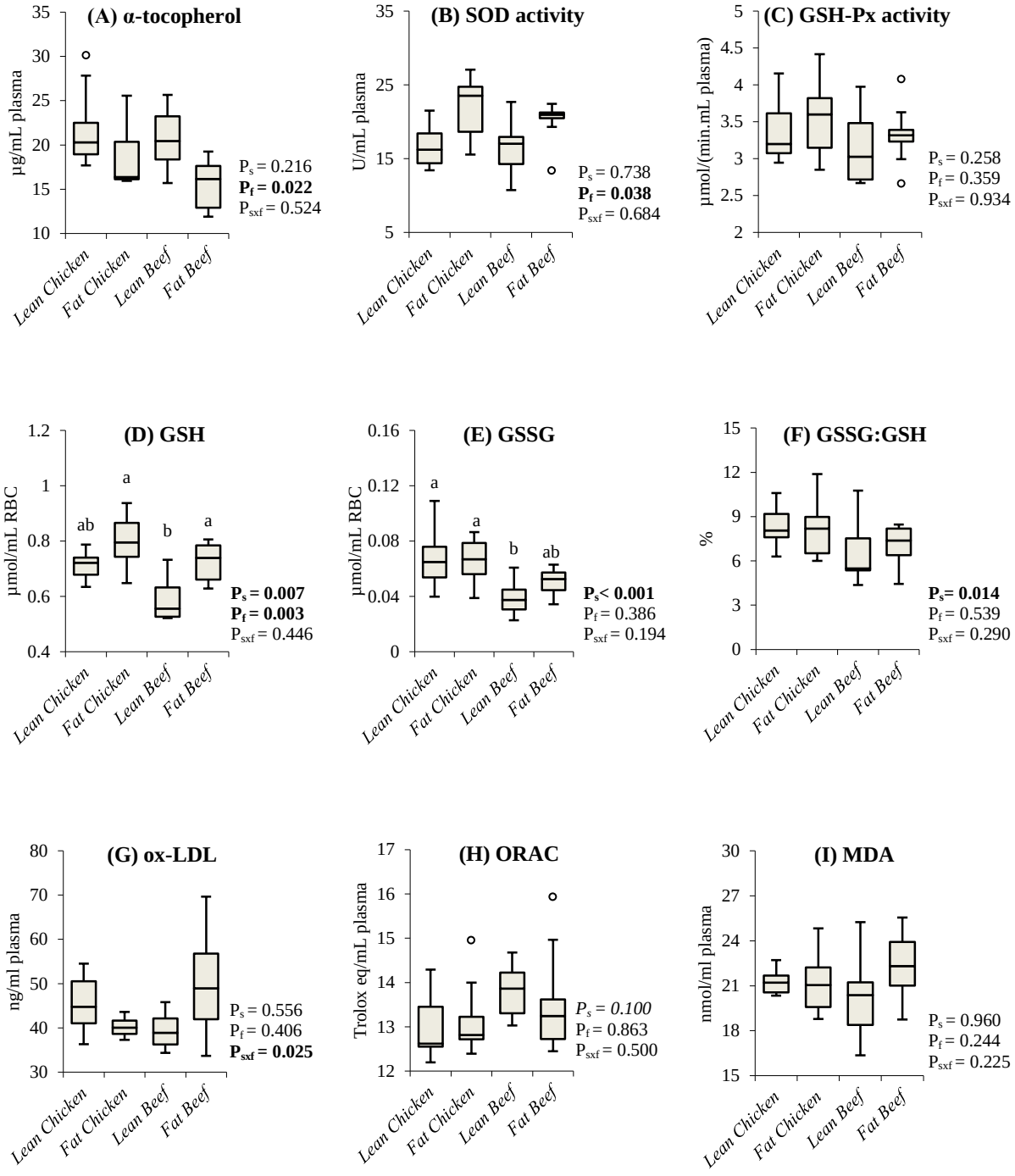

Figure 5.3: Oxidative parameters in rat plasma following 2 weeks meat diet; (A) α-tocopherol, (B) superoxide dismutase (SOD) activity, (C) glutathione peroxidase (GSH-Px) activity, (D) glutathione (GSH), (E) oxidized glutathione (GSSG), (F) oxidized glutathione ratio (GSSG:GSH), (G) oxidized low-density lipoproteins (ox-LDL), (H) oxygen radical absorbance capacity (ORAC), (I) malondialdehyde (MDA). Different superscripts indicate significant differences ($P<0.05$) among diets. Ps = P-value of the fixed factor species, Pf = P-value of the fixed factor fat content, Ps×f = P-value of the interaction term species × fat content, P-values < 0.05 are highlighted in bold, P-values between 0.05 and 0.10 are highlighted in italic.

Inflammation

C-reactive protein was significantly higher in plasma of rats on the lean beef diet compared to the lean chicken (+39.9%) and fat beef (+57.8%) diet, and tended to be higher compared to rats on the fat chicken diet (+21.3% , P=0.097) (Figure 5.4). C-reactive protein of rats on the fat chicken diet tended to be higher compared to the fat beef (+30.1%) diet (P=0.065).

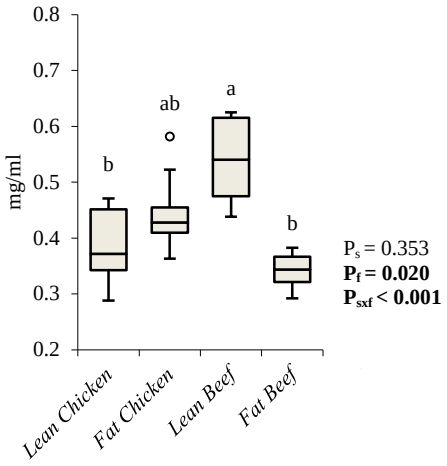

Figure 5.4: Low-grade inflammation in rats on the lean beef diet determined by C-reactive protein (CRP) in plasma. Different superscripts indicate significant differences (P<0.05) among diets. P_s = P-value of the fixed factor species, P_f = P-value of the fixed factor fat content, $P_{s\times f}$ = P-value of the interaction term, P-values < 0.05 are highlighted in bold.

Urinary parameters

1,4-dihydroxynonane mercapturic acid (DHN-MA) was increased in the urine of rats on the lean beef diet (Table 5.2). When expressed per 24h urinary excretion, TBARS was not different between the dietary treatments. Uric acid and allantoin were lower in urine of rats on the fat diets, whereas the beef diets tended to increase urinary allantoin. Also, the allantoin:uric acid ratio was clearly higher in the beef diets compared to the chicken diets. The beef diets resulted in higher urinary TMA and TMAO, with highest levels found in the urine of rats on the lean beef diets.

Table 5.2: Urinary parameters in rats following 2 weeks on the meat diets.

		Chicken		Beef			P-values		
	Unit	**Lean**	**Fat**	**Lean**	**Fat**	**SEM**	**P_s**	**P_f**	**$P_{s\times f}$**
DHN-MA	µg/24h	0.79^{b}	0.72^{b}	1.85^{a}	0.69^{b}	0.229	**.025**	**.010**	**.019**
TBARS	µmol/24h	0.21	0.20	0.22	0.21	0.017	.436	.541	.788
Uric acid	mg/24h	7.6	6.3	6.8	5.2	0.65	.150	**.036**	.833
Allantoin	mg/24h	105^{ab}	88^{b}	135^{a}	99^{ab}	10.7	*.051*	**.018**	.335
Allantoin:uric acid	-	13.8^{b}	14.0^{b}	19.9^{a}	19.0^{a}	1.17	**<.001**	.876	.448
TMA	µmol/24h	0.45^{c}	0.36^{c}	2.29^{a}	1.46^{b}	0.147	**<.001**	**<.001**	**.004**
TMAO	µmol/24h	2.75^{bc}	2.12^{c}	11.18^{a}	6.95^{ab}	1.254	**<.001**	*.062*	.157

DHN-MA= 1,4-dihydroxynonane mercapturic acid. TBARS= thiobarbituric acid reactive substances. TMA= trimethylamine. TMAO= trimethylamine-*N*-oxide. Different superscripts indicate significant differences ($P<0.05$) among diets. P_s = P-value of the fixed factor species, P_f = P-value of the fixed factor fat content, $P_{s\times f}$ = P-value of the interaction term, P-values < 0.05 are highlighted in bold, P-values between 0.05 and 0.10 are highlighted in italic.

Malondialdehyde in organs

The diets had a significant effect on the TBARS concentration in the sampled organs (Figure 5.5). Consumption of the beef diets resulted in a significantly higher TBARS concentration in the colonic tissues (+8.8%) compared to rats fed the chicken diets. Rats fed the fat beef diet had significantly higher TBARS values in heart tissue (+12.9%) compared to rats on the lean chicken diets, and also had higher kidney TBARS compared to all other groups (+24.4%) (all $P<0.05$). Further, rats on the fat diets had significantly lower TBARS levels in the brains (-6.8%) whereas in the prostate, TBARS levels tended to be higher (+14.5%). No significant effects were observed in the other sampled tissues (data not shown).

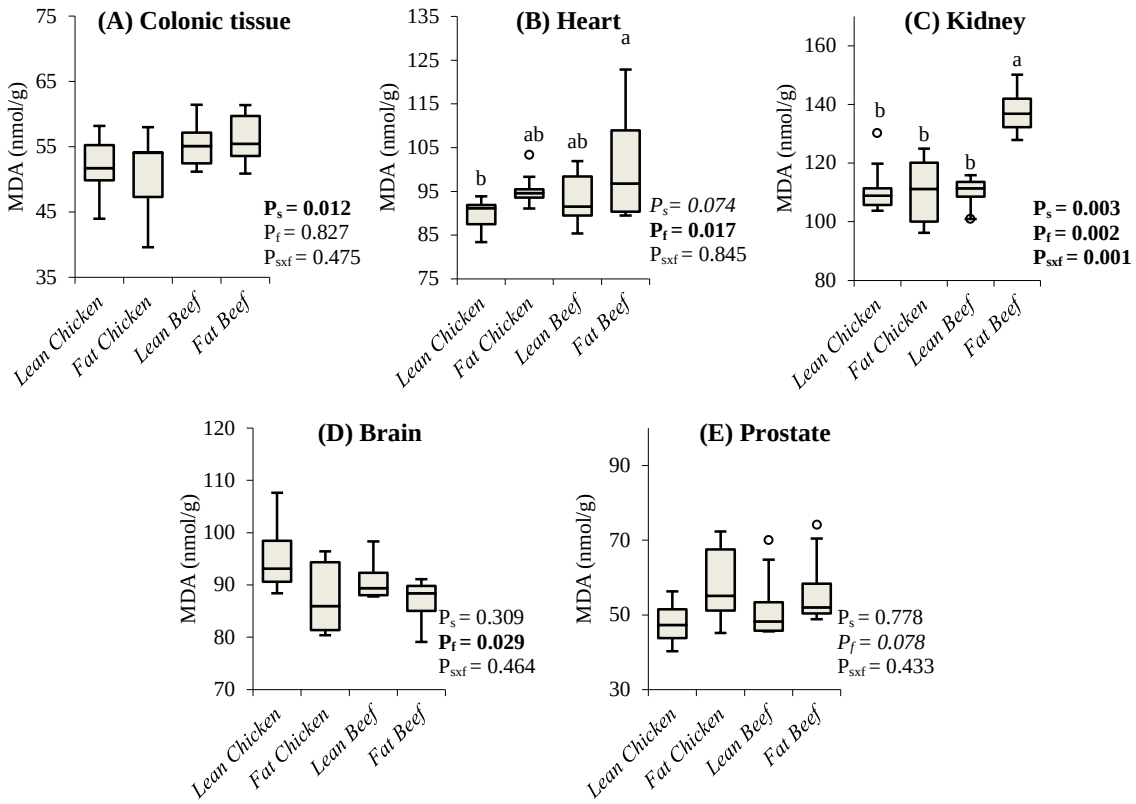

Figure 5.5: thiobarbituric acid reactive substances (TBARS) concentrations in selected organs of rats following 2 weeks consumption of the lean chicken-, fat chicken-, lean beef- or fat beef diet; (A) colonic tissue, (B) heart, (C) kidney, (D) brain and (E) prostate. Different superscripts indicate significant differences (P<0.05) among diets. Ps = P-value of the fixed factor species, Pf = P-value of the fixed factor fat content, Ps×f = P-value of the interaction term, P-values < 0.05 are highlighted in bold, P-values between 0.05 and 0.10 are highlighted in italic.

α-tocopherol in liver

Rats on the fat beef diet had significantly lower α-tocopherol concentrations in the liver (-26.2%) compared to the liver of rats on the lean chicken diet, and a trend (P=0.069) for lower levels compared to the fat chicken diet (Figure 5.6).

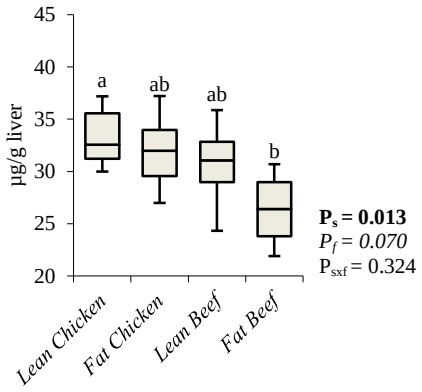

Figure 5.6: Concentrations of α-tocopherol in liver of rats following 2 weeks consumption of the lean chicken-, fat chicken-, lean beef- or fat beef diet. Different superscripts indicate significant differences (P<0.05) among diets. P_s = P-value of the fixed factor haem-Fe, P_f = P-value of the fixed factor fat content, $P_{s\times f}$ = P-value of the interaction term, P-values < 0.05 are highlighted in bold, P-values between 0.05 and 0.10 are highlighted in italic.

Glutathione peroxidase activity in organs

No significant effects were found between the different dietary treatments (Table 5.3).

Table 5.3: Glutathion peroxidase (µmol/(min.g)) in selected organs of rats following 2 weeks consumption of the experimental diets.

	Chicken		Beef			P-values		
	Lean	**Fat**	**Lean**	**Fat**	**SEM**	**P_s**	**P_f**	**$P_{s\times f}$**
Duodenum	3.6	3.6	3.4	3.8	0.16	.895	.186	.201
Colon	3.6	3.8	3.4	3.7	0.16	.472	.146	.887
Heart	13.9	13.7	13.3	13.6	0.37	.267	.780	.485
Kidney	17.4	18.7	18.2	18.9	0.83	.486	.169	.634
Liver	58.4	52.0	51.0	52.2	3.70	.312	.460	.281
Brains	0.94	0.99	0.99	0.93	0.03	.837	.864	*.078*

SEM= standard error of the mean, P_s = P-value of the fixed factor species, P_f = P-value of the fixed factor fat content, $P_{s\times f}$ = P-value of the interaction term, P-values between 0.05 and 0.10 are highlighted in italic.

Liver fatty acids

Rats on the fat diets had significantly higher concentrations of total FA and all of its subclasses (Table 5.4). Rats on the beef diets had significantly lower concentrations of total FA, MUFA, ALA and LA, whereas they had higher concentrations of long chain *n*-3 PUFA. Rats on the lean beef diet had 30.2% more hepatic DHA compared to rats on the lean chicken diet (P=0.015).

Table 5.4: Liver fatty acid composition of rats following 2 weeks consumption of the experimental diets.

		Chicken		Beef			P-values		
		Lean	Fat	Lean	Fat	SEM	P_s	P_f	$P_{s\times f}$
Total FA	g/100g	4.91[b]	8.10[a]	4.00[b]	7.13[a]	0.423	**.043**	**<.001**	.938
SFA	g/100g	1.64[b]	2.47[a]	1.52[b]	2.28[a]	0.122	.174	**<.001**	.711
MUFA	g/100g	1.71[b]	2.84[a]	1.22[b]	2.30[a]	0.193	**.017**	**<.001**	.887
PUFA	g/100g	1.55[b]	2.76[a]	1.26[b]	2.54[a]	0.146	.104	**<.001**	.792
LC *n*-3 PUFA	mg/100g	100[c]	184[a]	131[b]	192[a]	7.3	**.015**	**<.001**	.141
DHA	mg/100g	86[c]	144[a]	112[b]	146[a]	5.9	**.019**	**<.001**	**.034**
ALA	mg/100g	11[b]	49[a]	6[b]	38[a]	3.5	**.032**	**<.001**	.379
LC *n*-6 PUFA	mg/100g	591[b]	789[a]	586[b]	797[a]	25.2	.945	**<.001**	.805
LA	mg/100g	851[b]	1735[a]	538[b]	1517[a]	172.8	**.046**	**<.001**	.704

Total FA = total fatty acids; SFA=saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; LC *n*-3 PUFA = long chain *n*-3 polyunsaturated fatty acids (C20:5,*n*-3; C22:5,*n*-3; C22-6,*n*-3); DHA= docosahexaenoic acid; ALA = α-linolenic acid (C18:3,*n*-3); LA = linoleic acid (C18:2,*n*-6); LC *n*-6 PUFA = Long chain *n*-6 polyunsaturated fatty acids (C20:4,*n*-6; C22:4,*n*-6; C22:5,*n*-6); FA = fatty acid; Different superscripts indicate significant differences ($P<0.05$) among diets. P_s = P-value of the fixed factor species, P_f = P-value of the fixed factor fat content, $P_{h\times f}$ = P-value of the interaction term, P-values < 0.05 are highlighted in bold.

DISCUSSION

The present short-term rat feeding trial demonstrated that a high consumption of beef *vs.* chicken may increase oxidative stress parameters and inflammation, depending on the fat content of the diet. The meat diets not only affected oxidative stress parameters in gastrointestinal tract mucosae in direct contact with digested meat components, but also affected extra-gastrointestinal organs such as the heart, kidneys, brains and prostate. These data could contribute to understanding the mechanisms underlying the higher risk of developing a range of oxidative stress related diseases such as CRC (Chan et al., 2011) and coronary heart disease (Micha et al., 2012) with high consumption of red (processed) meat, observed in several large-scale meta-analyses of epidemiological studies.

Lipid oxidation occurred during digestion, observed by a pronounced MDA increase in the acid stomach, similar to previous observations (Gorelik et al., 2008a). Especially during the digestion of the beef diets, which had high haem-Fe contents, the TBARS increase was very evident.

Previously, we described the pro-oxidant effect of haem-Fe during the *in vitro* digestion of meat products (Chapter I). The considerably lower TBARS concentrations in the colonic content compared to the stomach content, may be explained by MDA absorption (Gorelik et al., 2008b) or degradation or metabolization of MDA into other compounds during colonic fermentation. Rats fed the beef diets showed increased TBARS concentrations in colonic tissues, likely due to the direct contact with the high TBARS-containing colonic beef digests. In agreement, chronic exposure to high levels of ferrous iron increased LOP in colonic mucosae of rats (Lund et al., 2001). Increased lipid oxidation in colonic tissues is one of the mechanisms proposed to be associated with increased CRC risk (Corpet, 2011). However, the direct involvement of MDA itself in CRC development may be questioned, since duodenal mucosae were exposed to up to 90% higher TBARS concentrations compared to the colonic mucosae, yet cancers occurring in the duodenum are extremely rare (Neugut et al., 1998). Possibly, oxidative stress following beef consumption alters the colonic microbial composition and hence their metabolic capacity, which might facilitate deleterious effects on colonic mucosae. Qiao et al. (2013) showed that MDA in the colonic content of mice was positively associated with the quantity of *E. coli* and *Enterococcus* spp., while negatively correlated with *Lactobacillus* spp. Ijssennagger et al. (2012) found similar microbial composition changes after feeding mice a haem-rich diet, accompanied with increased microbial nitrate-reducing capacity. This microbial composition change might be of importance since a high variation was observed in the potency of individual human fecal microbiota to generate NOC-derivative DNA adducts during *in vitro* digestion of meat (Vanden Bussche et al., 2014; Chapters I, II and III). Furthermore, ROS may activate other meat-related genotoxins such as benzo[a]pyrene (Dix & Marnett, 1983) and NOCs (Miura et al., 2011) to its ultimate carcinogenic form. Therefore, oxidative stress may have an indirect rather than a direct influence on CRC development.

Absorption of MDA in the bloodstream during meat digestion reaches a maximum 3 hours after consumption, after which it gradually decreases (Gorelik et al., 2008b). In the present study, rats were fed *ad libitum*, and had access to food until the moment of euthanasia as digestion samples had to be collected. Therefore, varying eating times could explain the absence of dietary effects on plasma MDA concentrations. The higher plasma SOD activity and GSH concentrations in rats on the fat meat diets could be an adaptation mechanism to the lower plasma vitamin E levels. Analysis of vitamin E in the liver confirmed that rats on the fat beef diet had low vitamin E levels. Levels of vitamin E in tissues and plasma are important in the overall antioxidant defense since vitamin E depleted animals are more susceptible to oxidative stress (Chow, 1991), while on the other hand low vitamin E levels can indicate increased vitamin E utilization (Esterbauer et al., 1992). The lower blood GSH in the lean beef group was accompanied with higher urinary DHN-MA. This observation most likely reflects that the latter metabolite is a mercapturic conjugate of the lipid peroxidation product 4-HNE with endogenous GSH (Alary et al, 1995). However, it is puzzling that these effects were not observed in rats on the fat beef diets. Possibly, the different fatty acid profiles between the lean and fat diets could contribute to the different effects. While the fatty acids in the lean diets mainly originated from safflower oil which is high in *n*-6 PUFAs, the contribution of safflower oil and *n*-6 fatty acids to total fat was considerably lower in the fat diets. Since 4-HNE only originates from *n*-6 fatty acids, and only 4-HNE, not MDA, reacts with GSH (Esterbauer et al., 1991) a different profile of formed LOP during digestion of the lean and fat beef diet might contribute to the observed differential effects on GSH. Also, rats on the fat diets consumed less of the diet which could also contribute to explaining the observed effects.

Rats on the beef diets had higher allantoin concentrations in urine, accompanied with a higher urinary allantoin:uric acid ratio. In humans, urinary allantoin is a potential marker for oxidative stress since the conversion of uric acid to allantoin in humans can only occur by ROS (Il'yasova

et al., 2012). However, in mammals other than humanoid primates, uric acid can also be converted enzymatically to allantoin. Hence, its presence in rats is not a specific parameter for oxidative stress. Nevertheless, oxidative stress may have contributed to the increased allantoin:uric acid ratio in rats on the beef diets.

High red meat consumption was associated with higher plasma CRP concentrations in epidemiological studies (Azadbakht & Esmaillzadeh et al., 2009; Montonen et al., 2013; Ley et al., 2014) suggesting a promotion of low-grade inflammation. In contrast, plasma CRP was not raised in humans when carbohydrates were replaced with lean red meat (~200g/day) in their diet for 8 weeks (Hodgson et al., 2007). In the present study, elevated CRP in the lean beef group was accompanied with lower GSH levels in blood and higher DHN-MA levels in urine, suggesting these alterations may contribute to the observed low-grade inflammation. However, when rats were fed a combination of haem-Fe and *n*-3 or *n*-6 PUFAs originating from fish oil and safflower oil respectively, plasma CRP remained equal but colonic myeloperoxidase activity as a marker of inflammation increased when haem-Fe was combined with fish oil (Guéraud et al., 2015). More research is warranted to elucidate the pro-inflammatory effects of red meat consumption and its likely interaction with other nutrients, since low-grade inflammation is associated with an increased risk to develop CRC, coronary heart disease and diabetes (Pradhan et al., 2001; Il'yasova et al, 2005).

Intriguingly, only rats on the fat beef diet, and not on the lean beef diet, had increased TBARS concentrations in the heart and kidneys, compared to rats on the lean chicken diet. The higher TBARS concentrations were accompanied with lower vitamin E concentrations in the liver. These findings could contribute to explaining the epidemiological outcome of the large scale meta-analysis (>1.2 million individuals) by Micha et al. (2010) who reported a 42% increased coronary heart disease risk per 50g serving of processed meat per day, but no increased risk when

consuming 100g serving of unprocessed red meat per day. Previous authors suggested the higher sodium and nitrite preservative levels in processed meat could explain these associations. Based on our observations, we hypothesize that high consumption of fat beef products may lead to higher coronary heart disease risk through a combination of several additional mechanisms (Figure 5.7). Oxidative stress in the renal medulla was previously described to induce hypertension by a variety of mechanisms among which renal vasoconstriction and renin release (Araujo & Wilcox, 2014). Therefore, the observed higher kidney TBARS in rats on the fat beef diet may be an indication of renal oxidative stress and may favor the onset of hypertension. In accordance, Lajous et al. (2014) reported an increased risk for hypertension in women when consuming large amounts of processed red meat, but not unprocessed red meat. Next to the elevated TBARS concentrations in kidneys, the fat beef diet also increased cardiac TBARS. Extensive evidence is available indicating a causative role for oxidative stress in the development of cardiac hypertrophy (Maulik & Kumar, 2012). Oxidative stress may result in oxidation of low-density-lipoproteins, which can lead to endothelial dysfunction and promote the formation and progression of atherosclerotic plaques (Mitra et al., 2011). Following a hamburger meal, humans showed an increase in ox-LDL (Ahotupa et al., 2010). Our results showed a significant interaction between meat species and fat content in the diet on ox-LDL concentrations. Not only the fat beef diet resulted in a non-significant ox-LDL increase, but also the lean chicken diet compared to the other two diets, which deserves further research. An important remark is that a high sucrose intake is also considered a risk factor for CVD, hypertension and kidney disease (Johnson et al., 2007). Therefore, it is possible that the effects of the high fat beef diet in this study were mediated or worsened by the simultaneous high sucrose intake. Indeed, heart tissues of rats on a 2-week high sucrose (65%) diet, had a higher susceptibility to peroxidation assays compared to rats on a starch diet (Busserolles et al., 2002).

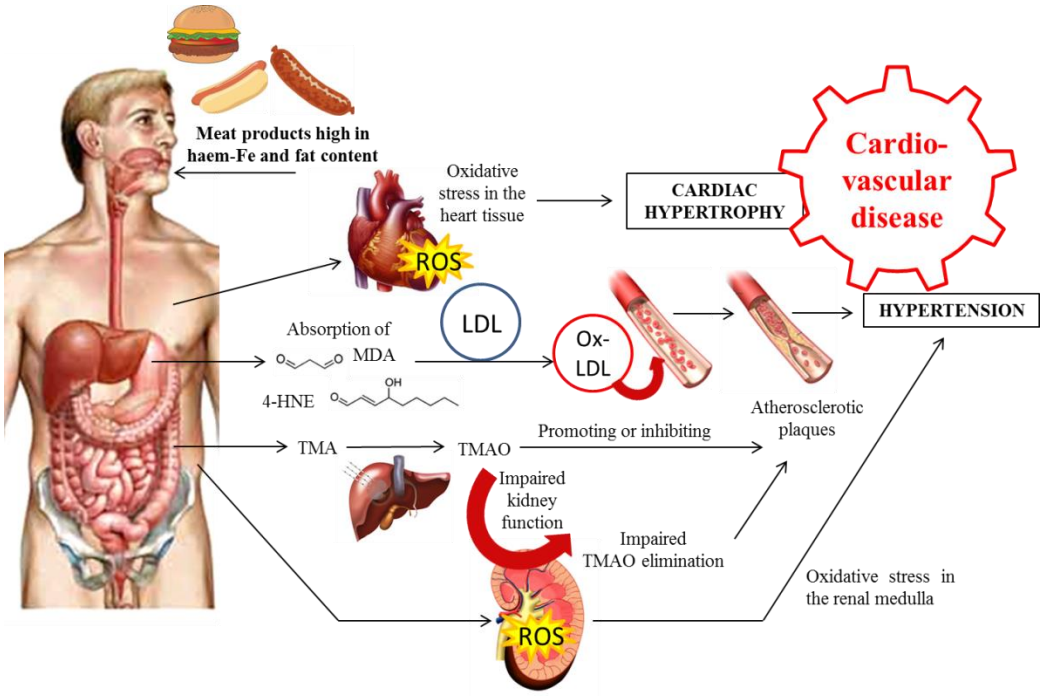

Figure 5.7: Proposed mechanism for the relation between high processed red meat consumption and cardiovascular disease.

In addition, an increase in urinary TMA and TMAO was observed in the present study when rats consumed the beef diets. These compounds were especially elevated in the urine of rats consuming the lean beef diet, corresponding to their higher intake of *L*-carnitine compared to the fat beef diets. Both proatherogenic (Koeth et al., 2013; Tang et al., 2015) and cardioprotective (Johri et al., 2014; Collins et al., 2016) properties of *L*-carnitine and TMAO have been described, which challenges its interpretation in relation to cardiovascular risk. However, if TMAO would represent the main mechanism explaining the association between high red meat consumption and CVD, it is puzzling why only processed meat, and not unprocessed red meat is associated with a higher epidemiologic risk to develop CVD (Micha et al., 2010) or hypertension (Lajous et al., 2014).

Epidemiological studies suggest a role of high fat diets in the development of prostate cancer (Mandair et al, 2014), but its impact is still a subject of discussion (Xu et al., 2015). Rats on the fat diets tended to have higher TBARS concentrations in the prostate and similarly, tended to have a higher ventral prostate weight. In accordance, a high fat diet previously induced an increased weight of the ventral prostate (Cai et al., 2001). Since oxidative stress is believed to exert an important role in the onset and progression of prostate cancer (Khandrika et al., 200), our results suggest that a high fat content in the diet, and not meat species or haem-Fe, may be related to the development of this disease.

In contrast to the other organs, a beneficial impact of the fat diets on the oxidative stability of the brains was observed. The essential fatty acid DHA is the precursor of the antioxidant neuroprotectin D1, which formation and presence only occurs in the brains and eyes (Bazan, 2005). Particularly rats on the lean chicken diet had a lower DHA intake compared to rats on the fat diets and accordingly, these rats had lowest hepatic DHA concentrations, followed by rats on the lean beef diet, whereas rats on the fat diets had highest hepatic DHA concentrations. Hence, it could be hypothesized that rats with a lower DHA status may be more prone to brain oxidative stress through lower formation of neuroprotection D1. Oxidative stress in the brains has been associated with several neurodegenerative diseases (Gandhi S & Abramov, 2012). No significant effects of the experimental diets were found on the other tested organs. Possibly, the duration of the feeding period of the experimental diets was not sufficiently long to detect differences in these organs.

In conclusion, in the present rat feeding intervention, we found stimulatory effects of beef diets on markers of oxidative stress and inflammation, and TMAO formation. Oxidation of meat during digestion may not only result in formation of LOP causing oxidative stress in the

gastrointestinal mucosae, but may also affect extra-gastrointestinal organs such as the heart and kidneys, potentially with deteriorate effects on health.

Chapter VI

Review: the role of red (processed) meat in the diet and *Helicobacter pylori* gastritis in oxidative stress-associated diseases

Adapted from:

Van Hecke, T., Van Camp, J., De Smet, S. The role of red (processed) meat in the diet and *Helicobacter pylori* gastritis in oxidative stress-associated diseases. *To be submitted.*

ABSTRACT

The last decade, epidemiologic evidence showing a positive association between high red meat and especially processed meat consumption with the risk to develop a range of chronic diseases is accumulating. Oxidative stress may be involved in these associations. However, oxidative stress is likely limited as long as red (processed) meat is consumed moderately and combined with "healthy" food choices such as a high consumption of vegetables and fruits, and low intake of refined sugars. Also, it seems likely that subgroups of the population are more prone to develop oxidative stress-related diseases as a consequence of high red (processed) meat consumption. For example, the inflamed stomach of *Helicobacter pylori* patients may be an excellent medium to enhance lipid oxidation following meat consumption. Oxidative stress resulting from red (processed) meat consumption may mediate the onset and/or progression of a wide range of diseases through various mechanisms discussed in the present review.

CONTENT

INTRODUCTION

As discussed previously, a wide range of epidemiological studies show that high red (processed) meat consumption is associated with a wide range of chronic diseases, among which CRC, coronary heart disease and diabetes (Figure 6.1). Although some plausible underlying mechanisms explaining these epidemiological observations were hypothesized over the years, the responsible mechanisms are still a matter of debate. However, diseases associated with high red (processed) meat consumption have one important condition in common; i.e. oxidative stress exerts a role in their etiology and/or progression (Valko et al., 2006; Chang et al., 2008; Sato et al., 2010; Rains & Jain, 2011; Chen & Keaney, 2012). Interestingly, infection of the stomach with *H. pylori*, which sensitizes to oxidative stress as discussed later on, is also associated with a higher risk to develop the aforementioned diseases in epidemiological studies. Moreover, the presence of these oxidative stress-related diseases are associated with each other in epidemiologic studies, indicating a common element in their etiology (Figure 6.1).

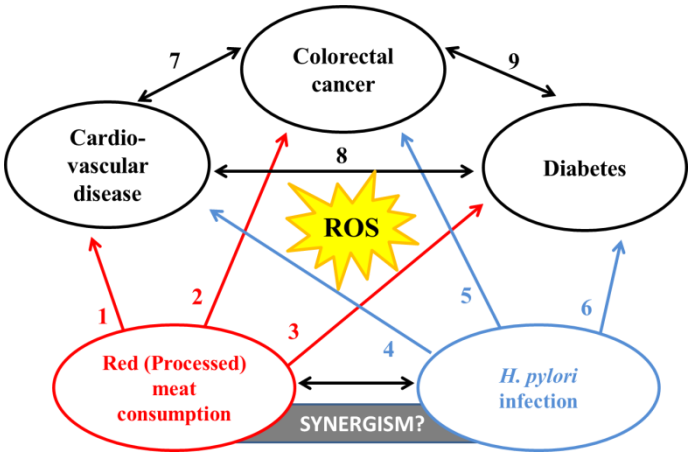

Figure 6.1: Epidemiologic associations between red (processed) meat consumption, *H. pylori* infection and oxidative stress-related diseases. (1) Red (processed) meat consumption – cardiovascular disease (Meta-analyses by Micha et al., 2010; 2012). (2) Red (processed) meat consumption – colorectal cancer (Meta-analyses by Chan et al., 2011; Aune et al., 2013; Xu et al., 2013). (3) Red (processed) meat consumption – diabetes (Meta-analyses by Micha et al., 2010; Pan et al., 2011). (4) *H. pylori* infection – cardiovascular disease (Meta-analyses by Sun et al., 2015; Liu et al., 2015). (5) *H. pylori* infection – colorectal cancer (Meta-analyses by Hong et al., 2012; Rokkas et al., 2013; Wu et al., 2013). (6) *H. pylori* infection – diabetes type II (Meta-analysis by Zhou et al., 2013). (7) Cardiovascular disease – colorectal cancer (Chan et al., 2006; Chan et al., 2007; Yang et al., 2010; Lee et al., 2013). (8) Cardiovascular disease – diabetes (Meta-analysis by Peters et al., 2014). (9) Diabetes mellitus – colorectal cancer (Meta-analyses by Jiang et al., 2011; Deng et al., 2012 ; De Bruijn et al., 2013).

In the first part of this review, we discuss how red (processed) meat related factors influence oxidation throughout digestion, and how other dietary compounds may inhibit or stimulate these reactions. Likely, the health risk of red (processed) meat consumption decreases when consumed in moderate amounts and part of a balanced diet high in vegetables and fruits, and low in sugars. We hypothesize high red (processed) meat consumption might especially be a risk factor for some population subgroups, for example patients with *H. pylori* infection or inflammatory bowel disease (IBD), which are common gastrointestinal diseases associated with oxidative stress. In the second part of the review, several mechanisms are discussed, explaining how oxidative stress following red (processed) meat consumption may contribute to disease development. We acknowledge that oxidative stress is likely not solely responsible for the associations made, but it

might be crucial in the onset and/or progression of these diseases. Finally, the meat industry is putting efforts to produce more "healthy" meat products, by addition of pure antioxidants or antioxidant-rich extracts/herbs/spices to meat products, *n*-3 fatty acid enrichment and reduction of residual nitrite. However, it should be avoided that these well-intentioned strategies are accompanied with increased susceptibility to LOP formation.

I. Red (processed) meat in the diet and oxidation during digestion

In the first part, the influence of various meat-related characteristics (*e.g.* haem-Fe, total fat content, fatty acid profile, nitrite, heating) and their interactions with other foods supplied through the diet (*e.g.* dairy products, vegetables, fruits, sugars) on the formation of ROS during the digestion of meat will be discussed (Figure 6.2). Likely, red (processed) meat digestion has differential effects on oxidation processes depending on the diets in which they are consumed, *e.g.* 'Western'-type diets which are typically high in sugars and fat, or 'prudent' diets high in antioxidants and fibers (Hu et al., 2000). The balance of compounds will determine if digestion of red (processed) meat will lead to ROS production, with fruits, vegetables and herbs as protective foods, and sugars as stimulating compounds. Some compounds such as calcium, nitrite and hydrophilic reducing compounds (*e.g.* ascorbic acid) may act both pro-oxidant and antioxidant, according to their concentrations and ratio to the present haem-Fe and ROS concentrations, as discussed later on. Furthermore, the inflamed stomach caused by *H. pylori* infection or an inflamed colon (inflammatory bowel disease) may stimulate the formation of oxidation products during red (processed) meat digestion.

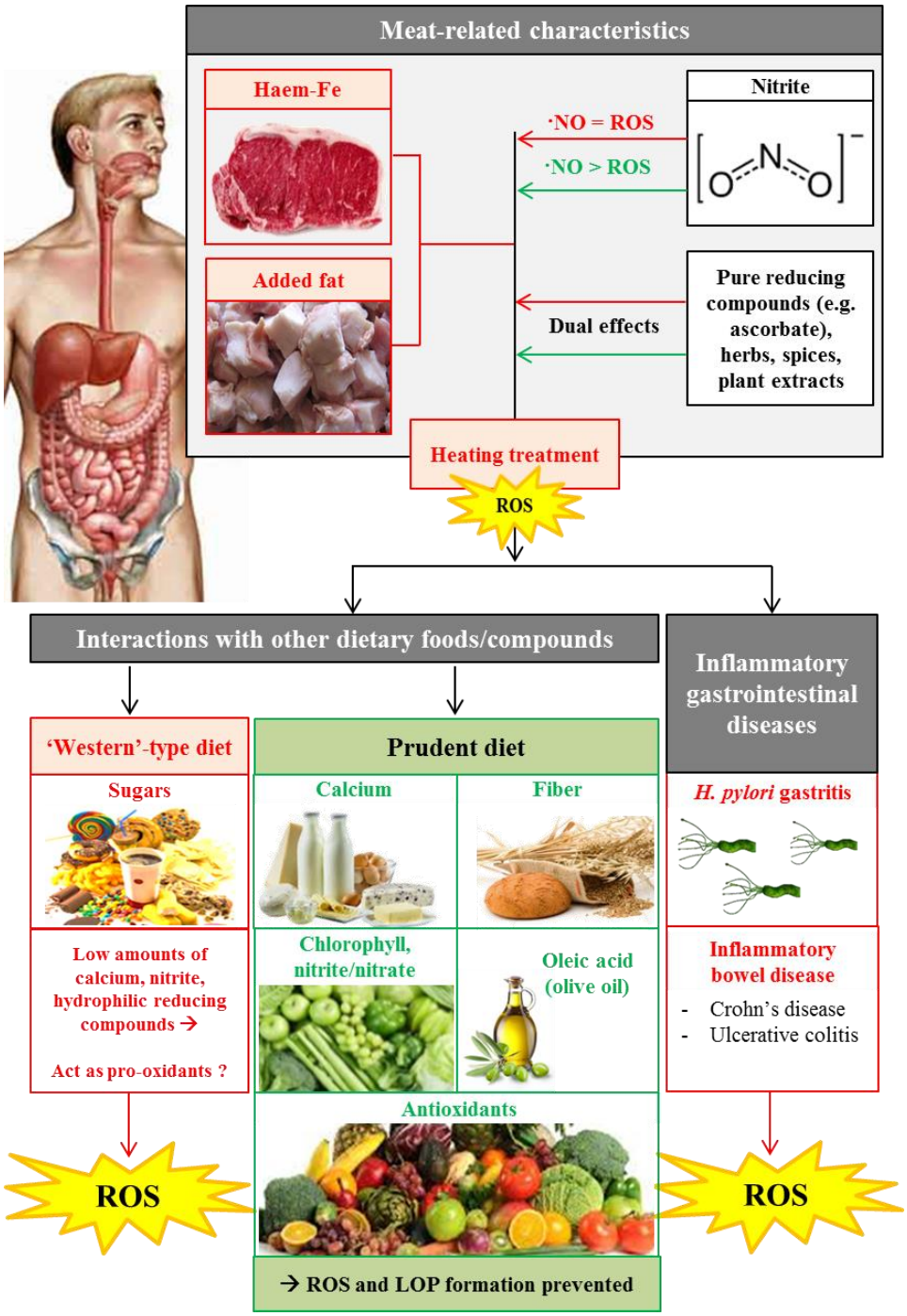

Figure 6.2: overview part I. Oxidation during red (processed) meat digestion is modulated by the characteristics of the meat product, other foods/compounds present in the diet, and is stimulated by inflammatory gastrointestinal diseases. Green and red arrows/boxes indicate antioxidant and pro-oxidant action respectively. ROS = reactive oxygen species. LOP = lipid oxidation products.

Meat-related characteristics affecting oxidation during digestion

Haem-Fe

Extensive evidence is available indicating haem-Fe in red (processed) meat as the key factor promoting oxidation. Muscles show a large variability in haem-Fe, according to the animal source and muscle type. White meat such as chicken has low haem-Fe contents (0.12-0.29 mg/100g), while both pork (0.20-0.32 mg/100g) and beef (1.68-2.11 mg/100g) are considered red meat, even though pork has considerately lower haem-Fe concentrations compared to beef (Lombardi-Boccia et al., 2002). In rats, dietary hemoglobin, beef meat and blood sausage were found to be associated with increased TBARS and urinary excretion of 1,4-dihydroxynonane mercapturic acid (DHN-MA), a urinary metabolite of 4-HNE (Pierre et al., 2004; Pierre et al., 2006). An increase in urinary DHN-MA was also observed in volunteers given a haem-Fe loaded blood sausage (Pierre et al., 2006). Dietary haem-Fe increased fecal TBARS in mice, accompanied with an oxidative stress response in the colonic mucosae (Ijssennagger et al., 2013). Rats consuming beef had higher plasma MDA concentrations (Toden et al., 2010), and higher TBARS in stomach- and colonic content and colonic tissues (Chapter V), compared to rats fed chicken meat. *In vitro* digestion of beef resulted in higher TBARS, 4-HNE and hexanal concentrations in duodenal and colonic digests compared to pork, followed by chicken meat (Chapter I). In accordance, *in vitro* digestion of beef resulted in higher protein oxidation compared to digested pork (Rysman et al., 2016).

Fat content and fatty acid profile

Next to the high haem-Fe content of meat, the presence of a high fat content is likely to result in an increased production of LOP. While haem-Fe is considered to facilitate oxidative reactions by stimulating the Fenton reaction, the presence of fat as a substrate is required to form LOP to a

large extent. Mixing muscle with subcutaneous pork fat is a common practice during processing of meat products. *In vitro* digestion of a heated model pork or beef product resulted in a clearly higher production of TBARS, 4-HNE and hexanal when lard was added and mixed with muscle, compared to heated lean muscle without added fat (1% fat) (Chapters II and IV). Steppeler et al. (2016) also reported increasing concentrations of MDA with increasing fat content in beef products during *in vitro* digestion.

Not only the total fat content of the meat product influences LOP formation, also the fatty acid profile was reported to be of importance in a recent rat feeding study (Guéraud et al., 2015) and *in vitro* digestion studies (Tirosh et al., 2015; Steppeler et al., 2016). Guéraud et al. (2015) showed urinary TBARS and DHN-MA in rats were not increased when a high haem-Fe content in the diet was combined with hydrogenated coconut oil (98.4% saturated fatty acids). When haem-Fe was combined with fish oil (predominantly *n*-3 fatty acids) or safflower oil (predominantly *n*-6 fatty acids), both TBARS and DHN-MA were increased. Urinary and fecal TBARS were highest when haem-Fe was combined with fish oil, while urinary DHN-MA was highest when haem-Fe was combined with safflower oil. Tirosh et al. (2015) and Steppeler et al. (2016) confirmed the importance of the fatty acid profile by showing increased MDA formation during *in vitro* digestion of red meat when combined with fish oil or an *n*-3 supplement. The addition of olive oil to red meat digestion resulted in lower MDA formation. Previously, it was reported that MUFAs such as oleic acid exert iron chelating properties (Balasubramanian et al., 1989). Tirosh et al. (2015) hypothesized that triglycerides of oleic acid, abundantly present in olive oil, would exert similar chelating behavior. In contrast, replacement of safflower oil with extra-virgin olive oil had no effect on fecal TBARS and urinary DHN-MA in rats given a beef diet (Pierre et al., 2008). Steppeler et al. (2016) reported that the amount of unsaturated fat, and not total iron content, was the dominating factor influencing the formation of aldehydes during *in vitro* digestion of chicken, pork, beef and salmon products with similar fat content. Indeed, a rat study conducted

by Guéraud et al. (2015) showed that rats consuming fish oil or safflower oil in combination with the catalytically inactive ferric citrate had higher urinary LOP compared to a haem-Fe / coconut oil diet. However, the haem-Fe / fish oil diet still resulted in approximately 3-fold higher urinary MDA concentrations compared to the ferric citrate / fish oil diet, and the haem-Fe / safflower diet even resulted in approximately 75-fold higher concentrations of urinary DHN-MA compared to the ferric citrate / safflower diet. Therefore, the presence of both haem-Fe and PUFAs are required for a maximal formation of LOP.

Heat treatment

Another factor contributing to increased oxidation during digestion is the heat treatment of meat before consumption. During heating of meat, Fe^{2+} is liberated through destruction of the heme-porphyrin moiety, oxymyoglobin releases O_2 with production of H_2O_2 and antioxidant enzymes (e.g. glutathione peroxidase) are inactivated (Kanner, 1994). These processes favor the stimulation of the Fenton reaction, which leads to a higher LOP formation. Rats consuming cooked meat products had increased fecal TBARS and urinary DHN-MA compared to rats consuming raw meats (Santarelli et al., 2010). Similarly, heating of a pork model product - whereby the core temperature was maintained 15 min at 65°C or 30 min at 90°C - increased the formation of TBARS, 4-HNE and hexanal before and during digestion. However, no relevant difference was observed between the 2 different heating procedures (Chapter III).

Nitrite

Next to haem-Fe and fat, nitrite is often present as an additive in processed meat products. Nitrite salt is widely used as a curing agent in meat products to inhibit outgrowth of *Clostridium*

botulinum, spoilage, and oxidative rancidity and to obtain the desired red meat color. The antioxidant mechanism of nitrite in meat was demonstrated by the group of Kanner (1994) and involves the antioxidant activity of the formed nitric oxide myoglobin, nitric oxide ferrous complexes, and *S*-nitroso-cystein and inhibition of the Fenton reaction. Furthermore, a stabilizing effect of nitrite was observed on the susceptibility of unsaturated lipids in the membranes to oxidation. In acidic conditions such as present in the stomach, nitrous acid generates dinitrogen trioxide (N_2O_3) and H_2O, which is in equilibrium with nitric oxide (•NO) and nitrogen dioxide ($•NO_2$) (Honikel, 2008). A dual role of •NO on lipid oxidation was described whereby a 1:1 ratio of •NO to ROS enhances lipid peroxidation through formation of $ONOO^-$ (peroxynitrite), whereas an excess of •NO inhibits oxidation (Darley-Usmar et al., 1995; Wink and Mitchell, 1998). A study using rats showed that nitrite-curing of meat products reduced TBARS in fecal water (Santarelli et al., 2010). Chenni et al. (2013) found that intake of nitrite through drinking water (1 g/l) reduced hemoglobin-induced lipid peroxidation in the colon of rats by 25%. This effect was not observed to be significant at lower doses (0.17 g/l nitrite and 0.23 g/l nitrate). During *in vitro* digestion of nitrite-cured meats (20g/kg nitrite salt corresponding to 120 mg nitrite/kg) varying in haem-Fe content, fat content or heating treatment, a drastic inhibition could be observed in the formation of LOP (Chapters I to III). However, this inhibition was less efficient when a high fat content was present (20% fat) or even absent when the meat products were subjected to the most intense heating procedure applied (core temperature maintained 30 min at 90°C). Interestingly, the latter meat products, in which nitrite was less efficient to prevent oxidant reactions, contained less residual nitrite. Therefore, the balance between •NO and ROS seems to be determining for the resulting outcome of nitrite on oxidant reactions.

In the last decades, when nitrite-curing is applied, sodium erythorbate, sodium ascorbate or ascorbic acid are added in typical concentrations of 500 mg/kg to meat products to decrease the

residual nitrite and the formation of nitrosamines (Honikel et al., 2008). The interactions of nitrite with ascorbic acid/ascorbate in meat products on the formation of LOP during digestion should be investigated in further experiments. Likely, the balance between nitrite, ascorbate and ROS determines the rate of gastrointestinal oxidation.

Interactions with dietary compounds affecting oxidation during digestion

Calcium

In 2003, Pierre et al. reported high dietary calcium (33.75 *vs.* 2.7g dibasic calcium phosphate/kg diet) inhibited hemoglobin-induced lipid peroxidation during digestion in rats, explained by precipitation of haem-Fe by calcium. Follow-up experiments confirmed that a high calcium supplement inhibited lipid peroxidation during consumption of a 60% beef diet (33.1g dibasic calcium phosphate/kg diet; inhibition fecal TBARS, no effect urinary DHN-MA) (Pierre et al., 2008), a 55% hotdog diet (15g calcium carbonate/kg diet; inhibition fecal TBARS, no effect urinary DHN-MA) (Santarelli et al., 2013) and a diet containing 55% cooked, nitrite-cured, dark meat (15g calcium carbonate/kg diet; inhibition fecal TBARS and urinary DHN-MA) (Pierre et al., 2013). However, Allam et al. (2011) found no decreased fecal TBARS when dibasic calcium phosphate (range 2-33 g/kg diet) was added to 60% beef-fed rats, even though haem-Fe was precipitated by doses ≥ 19 g/kg diet. Addition of calcium gluconate (106 g/kg diet) to the diet trapped haem-Fe, but also failed to lower fecal TBARS, while calcium carbonate (25 g/kg diet) both trapped haem-Fe and reduced fecal TBARS.

On the other hand, limited evidence is available describing the pro-oxidant action of calcium when added in low concentrations to various *in vitro* systems (Gutteridge, 1977; Savov et al., 1986;

Babizhayev, 1988). Previous authors suggested that low concentrations of Ca^{2+} are able to release Fe^{2+} bound to negatively charged lipid groups, hereby increasing the catalytically active Fe^{2+} and hence stimulating the Fenton reaction. In agreement, when chicken and beef muscle were injected with calcium chloride and cooked, the lower applied doses (0.05% for chicken; 0.05; 0.1; 0.15% for beef) were pro-oxidant after cooking the meat, while higher doses decreased lipid oxidation (Cho and Rhee, 1995). Injection of beef with calcium chloride (0.12; 0.24; 0.36%) and calcium lactate (0.24; 0.48; 0.72%) accelerated lipid oxidation, while calcium ascorbate (0.43; 0.86; 1.29%) inhibited (Lawrence et al., 2003). Similar to nitrite, the action of calcium during gastrointestinal oxidation could be dual, depending on its ratio to ROS and haem-Fe.

Reducing compounds

The simultaneous ingestion of foods rich in reducing compounds can be a successful strategy in lowering red (processed) meat-induced oxidative stress. The formation of oxidation products following a turkey meal in rats was inhibited by simultaneous ingestion of red wine polyphenols (Gorelik et al. 2008a). A similar TBARS reduction was observed in the stomach of mini-pigs, when a beef meal was consumed with a mixture of apples, artichokes and plums, or when combined with an extract of their phenolic compounds (Gobert et al., 2014). Also in humans, ingestion of red wine- (Gorelik et al., 2008b) and coffee polyphenols (Sirota et al., 2013) reduced plasma MDA following a turkey meal. Gorelik et al. (2013) showed LDL modification by MDA to be increased by 96% following 4 days of turkey meat consumption, which was completely prevented by simultaneous ingestion of red wine polyphenols. A spice mixture (45g/kg meat) was able to reduce TBARS formation during cooking of a high-fat beef product, and its consumption resulted in lower plasma and urine TBARS in humans compared to consumption of the meat product without herbs (Li et al., 2010). More studies reported on the inhibition of lipid oxidation

of turkey meat during *in vitro* gastric digestion by addition of caper (Tesoriere et al., 2007), melanoidins from traditional balsamic vinegar (Verzelloni et al., 2006) and from coffee, barley coffee and dark beer (Tagliazucchi et al., 2010). Antioxidants such as butylated hydroxyanisole (BHA), rutin, α-tocopherol, and carnosol lowered fecal TBARS and urinary DHN-MA in rats on a high hemin or high cured-meat diet (Pierre et al., 2003; Pierre et al., 2013).

However, the addition of reducing compounds seems to be a double-edged sword. Kuffa et al. (2009) found that addition of low concentrations of grape seed extract during simulated gastric digestion of high-fat (30%) turkey meat had a pro-oxidant effect, while higher concentrations exerted an antioxidant effect. In agreement with these findings, we recently observed that the concentration and partitioning behavior of reducing compounds and the initial oxidative status of meat products are determining factors in predicting the anti- or pro-oxidant outcome of added reducing compounds (Chapter IV). When low concentrations of a hydrophilic reducing compound (*e.g.* ascorbic acid and phenolic acids) are present during the digestion of a cooked high-fat beef product, the compound will partition in the water compartment, reducing Fe^{3+} to Fe^{2+}, and hereby stimulating the Fenton reaction and ROS production. Higher concentrations of hydrophilic reducing compounds decreased lipid oxidation, likely since they not only reduce Fe^{3+} to Fe^{2+}, but also scavenge ROS in the water compartment. On the other hand, lipophilic antioxidants (*e.g.* α-tocopherol) will concentrate in the fat compartment and will be able to efficiently neutralize ROS entering the fat compartment, hereby protecting the PUFAs from oxidation. Some reducing compounds such as caffeic acid and α-ferulic acid show amphiphilic behavior under acid conditions (Schwarz et al., 1996), and could hence exert a pro- or antioxidant behavior depending on their concentration, partitioning behavior and ratio to haem-Fe and ROS in the system. In contrast to the observations in the cooked high-fat beef product, the dual

effects of hydrophilic reducing compounds were not observed during digestion of a cooked low-fat beef product (Chapter IV). Tirosh et al. (2015) reported vitamin E to reduce lipid oxidation during digestion of red meat combined with olive oil (high in oleic acid). In contrast, vitamin E was pro-oxidant when added in a highly oxidized environment during the digestion of red meat in combination with fish oil (high in *n*-3 PUFAs). Hence the concentration and medium greatly affects the pro-/antioxidant outcome of added reducing compounds, however it is hypothesized that lipophilic reducing compounds are less susceptible to shift to pro-oxidant behavior.

Fibers

High consumption of resistant starch and fibers has been described to lower rectal DNA damage and attenuate colonic health parameters in humans and rats following red meat consumption (Paturi et al., 2012; O'Callaghan et al., 2012; Le Leu et al., 2015). However, few studies investigated the effect of fiber on oxidation of meat products during digestion. Hur et al. (2009) showed during *in vitro* digestion of a high-fat beef product that addition of 0.5% chitosan or pectin was able to reduce lipid oxidation, while addition of 0.5% cellulose had no effect. The authors explained this protective effect of chitosan and pectin since these fibers form a layer around the lipid droplets of the beef product, whereas addition of cellulose had no such effect. Simultaneously, addition of chitosan and pectin, resulted in a decreased concentration of free fatty acids compared to the control without fiber or with cellulose. Next to a direct protective effect of fibers against the formation of LOP, fibers may also indirectly protect against oxidative stress, since their fermentation leads to the formation of short-chain fatty acids (SCFA), among which butyrate. The latter is described as a protective compound lowering the risk to develop CRC (Fung et al., 2012). Daily rectal administration of butyrate to humans during 2 weeks

increased the colonic antioxidant glutathione (Hamer et al., 2009). Butyrate was also described to reduce H_2O_2 induced damage in human colonocytes (Rosignoli et al., 2001).

Chlorophyll

Chlorophyll is the pigment responsible for the color of green leafy vegetables (e.g. spinach and lettuce) and has a similar molecular structure as haem-Fe, with the important difference that chlorophyll has a non-reactive magnesium in the center of the porphyrin, instead of the highly reactive iron in haem-Fe. Addition of chlorophyll (12g/kg diet) to a diet supplemented with haem-Fe (326 mg/kg) reduced fecal TBARS in rats, while chlorophyllin, the water-soluble salt of chlorophyll, did not (de Vogel et al. 2005). Previous authors suggested the TBARS reduction was due to a sandwich formation of haem-Fe by chlorophyll, hereby blocking its chemical reactivity. The question remains if chlorophyll is still a protective compound after heating of the meat, when free reactive iron is released from the porphyrin.

Sugars

Addition of metal salts, among which $FeSO_4$, had little influence on autoxidation of methyl linoleate in an aqueous emulsion system at neutral pH. However, the addition of the metal salts in combination with fructose exerted a clear pro-oxidant behavior in the system, while this effect disappeared with simultaneous addition of metal chelators (Yamauchi et al., 1984; 1988). Therefore, the authors concluded that reducing sugars reduce transition metal ions, among which Fe^{3+} to Fe^{2+}, hereby accelerating the Fenton reaction and lipid oxidation. This mechanism might have important consequences when red (processed) meat is simultaneously consumed with sugar-rich foods such as sweetened drinks. Also, lipid oxidation and the Maillard reaction (browning

reaction between amino acids and reducing sugars) are so interrelated that they influence each other greatly (Zamora and Hidalgo, 2005). Unfortunately, to date, no studies are available studying the interaction between sugars and meat on oxidative stress parameters.

Short-term consumption of sucrose (disaccharide of glucose and fructose) was described to have a pro-oxidant effect on rats. Busserolles et al. (2002a; 2002b) reported decreased plasma vitamin E and lower heart SOD activity, and increased plasma and urinary TBARS in sucrose-fed rats, compared to starch-fed rats. This was accompanied by increased susceptibility of heart- and pancreas tissues to lipid peroxidation. The same group also showed that estradiol protected against sucrose-induced oxidation since the sucrose diet induced pro-oxidant effects in ovariectomized females, but not in intact females or ovariectomized females supplemented with estradiol (Busserolles et al., 2002c).

The inflamed gastrointestinal system

Helicobacter pylori

H. pylori is a widespread gram-negative bacteria, prevalent in one third of northern Europeans and northern Americans, and in more than 50% of the population in south and eastern Europe, South America and Asia (Eusebi et al., 2014). Infection causes chronic gastritis and despite the fact that the majority of infections remain asymptomatic, it is one of the leading factors for the development of gastric carcinoma (Wang et al., 2014). The clinical outcome of *H. pylori* infection might be associated with the presence of specific virulence-associated genes, of which the

distribution varies geographically. For example the East Asian genotype CagA (cytotoxic-associated gene product A) is predominant in populations with high incidence of gastric cancer, while the Western genotype CagA is predominant in Europe, Africa and South Asia, where incidence of gastric cancer is rather low (Yamaoka et al., 2008).

The *H. pylori*-infected stomach is characterized by gastric fluid containing decreased levels of vitamin C (Zhang et al., 1998), decreased synthesis of mucin (Byrd et al., 2000) which exerts antioxidant properties (Grisham et al., 1987) and increased ROS levels (Handa et al., 2010), mainly caused by activated neutrophils in the inflamed stomach (Figure 6.3). Infection with some *H. pylori* genotypes, such as $CagA^+$ strains, leads to higher ROS production in the stomach compared to infection with $CagA^-$ strains, induced by a higher neutrophil respiratory burst (Papa et al., 2002; Ladeira et al., 2008). In accordance, $CagA^+$ patients had lower gastric fluid vitamin C compared to $CagA^-$ patients (Zhang et al., 1998). In the acute phase of infection, gastric pH increases but decreases again to its normal levels within several months (McGowan et al., 1996). Therefore, a stomach infected by *H. pylori*, and likely $CagA^+$ strains in particular, may act as an excellent bioreactor stimulating oxidation. *H. pylori*-patients have increased blood MDA, ROS production, oxidative damaged DNA and SOD activity, and lower vitamin C concentrations compared to control subjects, indicating *H. pylori*-patients are under systemic oxidative stress (Khanzode et al. 2003; Mashimo et al., 2006; Siomek et al., 2006; Tiwari et al., 2010). The higher ROS production in peripheral blood of *H. pylori* patients was normalized after eradication of *H. pylori* (Mashimo et al., 2006). There are even indications that oxidative stress in gastric mucosae is present in asymptomatic *H. pylori*-patients, which is normalized after *H. pylori* eradication (Felley et al., 2002). As mentioned before, a 1:1 ratio of •NO to ROS enhances lipid peroxidation, whereas an excess of •NO inhibits oxidation (Darley-Usmar et al., 1995). In this regard, the inflamed stomach with excess of ROS could alter the •NO:ROS balance whereby nitrite in processed meat could act as a pro-oxidant. Consumption of red (processed) meat products,

which are already pro-oxidant by itself, may hence lead to enhanced oxidative stress in *H. pylori*-patients, compared to healthy subjects.

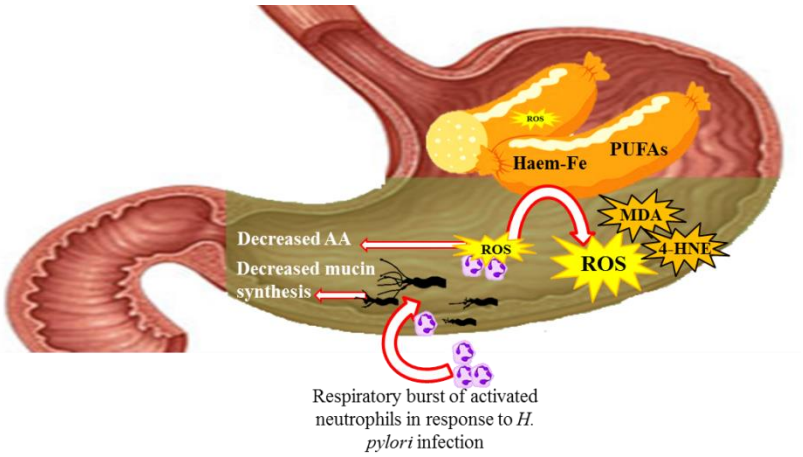

Figure 6.3: The respiratory burst of activated neutrophils in response to the *H. pylori* infected (especially with $CagA^+$ strains) stomach leads to higher ROS levels in the gastric juice, which will decrease gastric ascorbic acid (AA) by oxidation. Furthermore, *H. pylori* decreases synthesis of gastric mucin, which has protective and antioxidant properties. The pro-oxidant environment in the inflamed stomach can be synergistic or exacerbate the formation of lipid oxidation products such as malondialdehyde (MDA) and 4-hydroxy-nonenal (4-HNE) following red (processed) meat consumption.

Limited studies investigated the epidemiologic interaction between diet and *H. pylori* infection on disease risk (González et al. 2006, 2010, Epplein et al., 2014). These studies showed that the positive association between processed meat and gastric non-cardia cancer seemed to be restricted to *H. pylori*-patients. Unfortunately, no experimental studies are available studying the effect of meat consumption by *H. pylori*-patients on oxidative stress markers.

It must be kept in mind that *H. pylori*-patients are usually iron deficient (Annibale et al., 1999). The iron-regulatory hormone hepcidin may exert a central role in the etiology of this iron deficiency since hepcidin interferes with iron absorption and induces iron trapping in

macrophages following contact with pro-inflammatory stimuli such as lipopolysaccharides or interleukin-6 (Ganz, 2005). Therefore, depriving iron-deficient *H. pylori*-patients from unprocessed red meat might not be a good strategy, but it seems advisory to avoid processed meat at least until eradication and normalization of the stomach. Furthermore, especially in these patients, red meat consumption should be acompanied by a high intake of protective compounds.

Inflammatory bowel disease (IBD)

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory diseases of the intestines, associated with oxidative stress (Rezaie et al., 2007; Piechota-Polanczyk and Fichna, 2014). Even though CD and UC-associated CRC accounts for only 1-2% of all CRC cases in the general population, it is a serious complication of the disease and is responsible for 15% of the mortality in IBD patients (Munkholm, 2003). Moreover, IBD is associated with an increased incidence of cardiovascular events (Yarur et al., 2011; Rungoe et al., 2015). Both CD and UC patients are reported to have lower antioxidant concentrations in plasma, higher oxidative DNA damage in lymphocytes and upregulated plasma antioxidant enzymes (D'Odorico et al., 2001; Koutroubakis et al., 2004; Dincer et al., 2007). Carrier et al. (2001) showed that 0.3% iron in the diet increased plasma and colonic lipid peroxides in colitis-induced rats. Oral intake of ferrous fumarate also increased oxidative stress parameters in plasma of Crohn patients (Erichsen et al., 2003). Le Leu et al. (2013) reported an aggravation of colitis in mice when given red meat, but unfortunately no data on oxidative stress parameters were reported. Consequently, these data raise the question whether red (processed) meat consumption might exacerbate oxidative stress in these patients. But, as is the case with *H. pylori* infection, iron deficiency is a common side effect

in IBD patients (Kulnigg and Gasche, 2006). Similar dietary advice concerning red (processed) meat consumption for gastritis patients, could be given to these patients as well.

II. Implications of oxidation during digestion of meat on disease development

In the second part of the review, several mechanisms are discussed on how oxidative stress may contribute to the onset and/or progression of CRC, cardiovascular disease and diabetes, with special attention to red (processed) meat consumption, other dietary compounds and *H. pylori* infection (Figure 6.4). Likely, the mechanisms discussed are not exhaustive, but these are considered to be of major importance by the authors of this review.

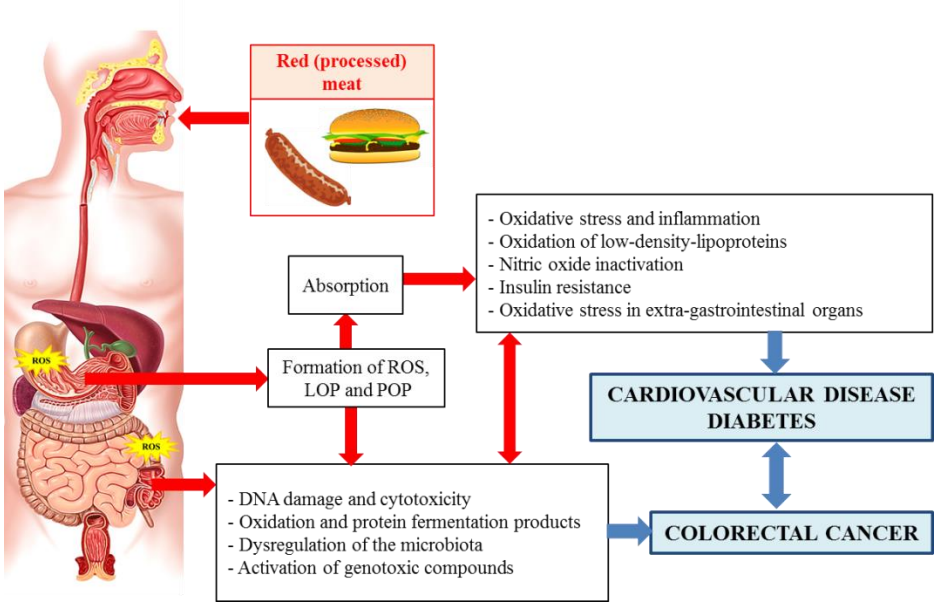

Figure 6.4: overview part II. Implications of oxidation during digestion of red (processed) meat on disease development. ROS = radical oxygen species, LOP = lipid oxidation products, POP = protein oxidation products.

DNA damage and cytotoxicity

Reactive oxygen species and LOP such as MDA and 4-HNE are described to induce DNA damage by adduct formation. Several DNA adducts as a consequence of oxidative stress are described in the literature such as pyrimido[1,2-α]purine-10(3H)-one-2'-deoxyribose (M_1dG; DNA adduct with MDA) and 1,N^6-etheno-2'-deoxyadenosine (εdA; DNA adduct with 4-HNE). The chemistry and formation of these DNA adducts will not be discussed here but is referred to reviews by Nair et al. (2007). In cell lines with normal expression of the adenomatous polyposis coli (Apc) tumor suppressor gene, 4-HNE and 4-HHE were more cytotoxic compared to Apc-deficient cell lines, and only genotoxic in the normal Apc cell line (Pierre et al., 2006; Baradat et al., 2011; Bastide et al. 2015). The mechanisms on the effects of LOP on cell signaling and cell death were reviewed by Guéraud et al. (2010) and will not be discussed here. Digests of a high-fat beef product were far more cytotoxic on CRC cell lines compared to digests of a low-fat beef product, while this was not attenuated by addition of antioxidants (Chapter IV). If DNA damage induced by LOP would be the main mechanism in which CRC is promoted as a consequence of high red (processed) meat consumption, it is however puzzling why red (processed) meat consumption does not promote cancers of the small intestines. Indeed, the presence of TBARS and other aldehydes peaks following the acid stomach digestion, as shown both *in vivo* (Chapter V) and *in vitro* (Chapters I to III). After all, MDA and 4-HHE are at least partly absorbed before they reach the colon (Gorelik et al., 2008b; Awada et al., 2012), and may be degraded or metabolized during further colonic digestion. However, oxidation could contribute to CRC through some other indirect mechanisms.

Dysregulation of the microbiota

Oxidative stress might modulate the microbiota, which is considered an important metabolic organ with an important impact on health and disease (O'Hara et al., 2006). Ijssennagger et al. (2012) showed a distinctive shift in the colonic microbial composition of mice fed a 'Western' diet (40% fat) supplemented with 0.5 μmol/g haem-Fe for 2 weeks, compared to mice fed the same diet without haem-Fe. The haem-Fe diets resulted in higher amounts of *Bacteroidetes* and lower amounts of *Firmicutes*. The authors argued this bacterial shift was most likely caused by a selective susceptibility of $Gram^+$ bacteria to haem-Fe cytotoxic fecal water, which was not observed for $Gram^-$ bacteria, thus favoring development of the latter community. An altered colonic redox state caused by high haem-Fe consumption might be a contributing explanation. The haem-Fe fed mice had 2.7 times more fecal MDA compared to the control mice. Another study by Qiao et al. (2013) showed mice fed a high-fat diet compared to a lean control diet had increased MDA concentrations in colonic contents, which was strongly positively associated with *E. coli* ($Gram^-$) and *Enterococcus* spp. ($Gram^+$) and negatively associated with *Lactobacillus* spp. ($Gram^+$). In accordance, Zimmer et al. (2012) observed higher counts of *Bacteroides* spp. ($Gram^-$), *Bifidobacterium* spp. ($Gram^+$), *E. coli* ($Gram^-$) and *Enterobacteriaceae* spp. ($Gram^-$) in omnivorous fecal samples compared to vegan samples, whereas subjects on a vegetarian diet ranked between vegans and controls.

A haem-Fe- or oxidative stress-induced microbial shift might be of importance since large inter-individual variation was observed in the ability of different individual porcine and human microbiota to form NOCs or NOC-derivative DNA adducts (Engemann et al., 2013; Vanden Bussche et al., 2014). In accordance, haem-Fe stimulated the formation of NOC-derivative DNA adducts during *in vitro* fermentation of meat digests (Chapter I; Vanden Bussche et al., 2014). Accompanied by the haem-Fe induced microbial shift, Ijssennagger et al. (2012) also observed an

increased nitrate reducing capacity of the colonic microflora, which could facilitate NOC formation. Possibly, the microbial composition might be the determining factor in the production of toxic NOCs by its nitrate reducing capacity. Also, alteration of the microbial communities following haem-Fe supplementation led to hyperproliferation and hyperplasia of the colonic epithelium (Ijssennagger et al., 2015), and alterations in the microbial communities could alter immunity and contribute to disease development (Belkaid et al., 2014). Manipulation of the intestinal microbiota altered the development of colitis-associated CRC in mice (Uronis et al., 2009), while depletion of luminal iron altered the gut microbiota and prevented the development of chronic ileitis in a murine model of Crohn's disease (Werner et al., 2010).

Activation of genotoxic compounds

During consumption of meat, other genotoxic compounds apart from LOP can be ingested or formed endogenously. Polycyclic aromatic hydrocarbons and HCAs are environmental toxicants and can be produced during intense heating of meat (Cross and Sinha, 2004). Consumption of red meat also leads to the endogenous formation of NOCs (Bingham et al., 2002; Lewin et al., 2006). Previous compounds are not genotoxic as such, but need a metabolic activation to exert their genotoxic activity (Demeyer et al., 2015). Next to an enzymatic activation (*e.g.* by cytochrome P450) of PAHs, HCAs and NOCs, also activation mechanisms have been described by Fenton oxidation. Early research by Dix and Marnett (1983) showed that oxidizing agents generated during lipid oxidation are able to epoxidize benzo[a]pyrene, a common PAH, to its carcinogenic form. Oxidation can also activate nitrosamines, leading to the formation of α-hydroxy nitrosamines, which spontaneously decompose to form an alkyl diazonium ion and a free alkyl carbocation, able to alkylate DNA (Grisham et al., 2000). Inami *et al.* (2010) showed the formation of direct-acting mutagens when *N*-nitrosodipropylamine (NDPA), *N*-

nitrosodibutylamine (NDBA), *N*-nitroso-*N*-methylpropylamine (NMPA) or *N*-nitroso-*N*-methylbutylamine (NMBA) were incubated with a hydroxyl radical generating system, consisting of Fe^{2+}, Cu^{2+} and H_2O_2, while this was not the case for *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA). In agreement, Mestankova et al. (2014) reported that oxidation of NDPA and *N*-nitrosopyrrolidine (NPYR) by hydroxyl radicals resulted in the formation of direct mutagens, while oxidation products of NDMA and NDEA were not. When Fe^{2+}, Cu^{2+} or H_2O_2 were removed from a reaction mixture with *N*-nitroso-*N*-methylpentylamine (NMPeA), the mutagenicity of the mixture decreased (Miura et al., 2011). Chen and Chung (1996) showed epoxidation of 4-HNE by fatty acid hydroperoxides and H_2O_2 to its mutagenic epoxide. The activation of genotoxic compounds by oxidative stress could contribute to explaining why consumption of chicken, despite containing considerable amounts of HCAs and PAHs (Skog et al., 1997; Kazerouni et al., 2001), is not associated with a higher risk of CRC in epidemiological studies since the extent of oxidation during digestion is very low compared to pork and beef products (Chapter I).

Oxidation and protein fermentation products

Lipid oxidation during processing of meat products or during its digestion stimulates oxidation of meat proteins (Utrera et al., 2014; Soladoye et al., 2015; Chapter II), which can be inhibited by antioxidants such as quercetin (Chapter IV). Oxidation of protein reduces its *in vitro* digestibility (Santé-Lhoutellier et al., 2007; Lund et al., 2011; Rysman et al., 2016) and thus it could be hypothesized that protein oxidation results in a higher amount of protein reaching the colon, subsequently leading to increased formation of potentially harmful protein fermentation products. In mini-pigs (Bax et al., 2013) and humans (Oberli et al., 2015), different heat treatments of consumed lean beef muscle slightly affected its ileal protein digestibility. The

different meat samples in the human study however had similar protein oxidation modifications, while the mini-pig study did not report on this. Possibly, heated high-fat meat products with a higher variation in protein oxidation could lead to increased differences in ileal digestibility. As mentioned before, *H. pylori* increases gastric pH in the acute phase of infection, but decreases again to its normal levels within several months (McGowan et al., 1996). Since gastric acid suppression impairs protein digestibility and increases the formation of colonic fermentation products in humans (Evenepoel et al., 1998), *H. pylori* patients might be exposed to higher concentrations of protein fermentation products. Moreover, the lower protein digestibility might be exacerbated by high oxidation in the inflamed stomach. More research is warranted to clarify the effect of protein oxidation on *in vivo* digestibility and its impact on the formation of colonic fermentation products and health issues.

Oxidative stress and inflammation

Oxidative stress is reported to be linked with inflammation. For the mechanisms involved, we refer to reviews covering this topic (Reuter et al., 2010; Gill et al., 2010). High red meat consumption was associated with higher plasma C-reactive protein (CRP) concentrations in epidemiologic studies (Azadbakht and Esmaillzadeh, 2009; Montonen et al., 2013; Ley et al., 2014), suggesting red meat consumption promotes low-grade inflammation. In contrast, plasma CRP was not raised in humans whereby carbohydrates were replaced with lean red meat (~200g/day) for 8 weeks (Hodgson et al., 2007). When rats were fed a combination of haem-Fe and PUFAs of different dietary origin, plasma CRP remained equal. However, colonic myeloperoxidase activity as a marker of inflammation increased when haem-Fe was combined with fish oil, accompanied with higher fecal TBARS (Guéraud et al., 2015). In agreement, a diet with oxidized *n*-3 PUFAs for 8 weeks increased plasma 4-HHE and inflammation in mice,

compared to the non-oxidized diets (Awada et al., 2012). Following a single consumption of Wagyu beef, CRP, TNF- α and IL-6 clearly increased, whereas the increase was less pronounced following consumption of kangaroo meat (Arya et al., 2010). In contrast, a single consumption of beef steak prepared either pan-fried or sous-vide had no impact on CRP or IL-6 in healthy young males. Following the pan-fried beef meal, TNF-α and MCP-1 remained equal, but these inflammatory markers decreased following the beef prepared sous-vide (Nuora et al., 2015). Recently, we observed elevated plasma CRP levels in rats consuming lean beef, but not when consuming fat beef, lean chicken or fat chicken in the context of a high-sugar diet (Chapter V). In this study, elevated CRP in the lean beef group was accompanied with lower blood GSH and higher urinary DHN-MA levels, suggesting increased 4-HNE concentrations might be associated with the observed inflammation. More research is warranted to elucidate the influence of meat consumption and its interactions with other dietary ingredients on inflammatory pathways.

Oxidation of low-density-lipoproteins

Oxidized low-density lipoproteins (ox-LDL) have a major role in the onset and progression of atherosclerosis by promoting endothelian dysfunction, leading to an inflammatory response (Mitra et al., 2011). Recently, it was shown in a cross-sectional study that subjects consuming high amounts of red meat had higher plasma ox-LDL, while this was not the case for high white meat consumption (Cocate et al., 2015). Following a hamburger meal, humans showed an increase in ox-LDL while LDL, HDL and ox-HDL remained equal (Ahotupa et al., 2010; Tiainen et al., 2014). Gorelik et al. (2013) confirmed ox-LDL increased in healthy volunteers following a red turkey meal. This formation of ox-LDL was completely prevented when the meal was combined with red wine polyphenols, which was accompanied with lower plasma MDA. Consumption of red wine (including the ethanol) also prevented the formation of cholesterol oxidation products

following the consumption of a cheeseburger (Natella et al., 2011). Also, humans having higher HDL concentrations are less susceptible to LDL oxidation following a hamburger meal (Ahotupa et al., 2010; Tiainen et al., 2014). This makes sense since HDL have antioxidant properties and are inversely associated with atherosclerosis (Bandeali and Farmer, 2012).

Previously, we hypothesized *H. pylori* patients would be more prone to red (processed) meat-induced oxidative stress. In this regard, HDL was decreased in *H. pylori* patients (Hoffmeister et al., 2001), which was normalized after *H. pylori* eradication (Scharnagl et al., 2004). Since high HDL prevents oxidation of LDL, it can be assumed that *H. pylori* patients are more prone to LDL oxidation following red (processed) meat consumption and therefore are at higher risk to develop atherosclerosis and heart disease. Indeed, among patients with coronary heart disease, individuals infected with $CagA^+$ *H. pylori* strains had higher ox-LDL concentrations and severity of coronary atherosclerosis, compared to the uninfected group and the $CagA^-$ group (Huang et al., 2011).

Nitric oxide inactivation

Under physiologic conditions, eNOS (endothelian nitric oxide synthase) produces nitric oxide (•NO), which is assumed to protect the cardiovascular system through various mechanisms, among which regulation of vessel dilatation and cardiac contraction (Lei et al., 2013). Under oxidative stress conditions, eNOS-derived •NO concentrations decrease through malfunctioning of eNOS and direct reaction of superoxide with •NO to the formation of peroxynitrite. The latter metabolite is able to oxidize the eNOS cofactor (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), leading to BH_4 deficiency (Li et al. 2014). Genetically engineered mice lacking eNOS developed

hypertension, atherosclerosis, insulin resistance and many more pathological features (Tsutsui et al., 2006). Although nitrate and nitrite used to be considered environmental pollutants and potential carcinogens, nowadays dietary nitrate and nitrite are even suggested to contribute to cardiovascular health by maintaining the pool of available •NO (Lundberg et al., 2010; Bondonno et al., 2015). Dietary nitrate/nitrite was described to reduce oxidative stress, inhibit inflammation, attenuate hypertension and improve several other cardiovascular parameters in different animal models and human volunteers (Bryan et al., 2008; Webb et al., 2008; Stokes et al., 2009; Carlström et al., 2011).

Micha et al. (2012) suggested the presence of nitrite in processed meats could partly explain the positive epidemiologic association between high processed meat and coronary heart disease and diabetes. The authors of the present review doubt the involvement of nitrite-curing in this association, since nitrite is physiologically present in saliva, its intake through meat is negligible when compared to nitrate/nitrite intake through vegetables (Temme et al., 2011), and as mentioned above, intake of nitrate/nitrite might even be beneficial for the cardiovascular system by increasing •NO availability. However, oxidative stress following red (processed) meat consumption may decrease plasma •NO concentrations, disturbing the regulation of the cardiovascular system. A high consumption of sucrose or fructose may have a similar mechanism since fructose has the unique ability among sugars to increase plasma uric acid, which is able to irreversibly react with •NO, resulting in the formation of 6-aminouracil and depletion of •NO (Johnson et al., 2007; Gersch et al., 2008; Kanbay et al., 2013). It is reasonable to assume that the impact of red (processed) meat consumption on plasma •NO levels at least partly depends on the interactions with other dietary compounds, with *e.g.* fructose as an aggravating factor and foods rich in antioxidants as protective factors.

H. pylori patients show higher plasma MDA but also higher •NO levels (Tiwari et al., 2010), which seems contrasting with the previous statements. However, due to inflammation,

expression of iNOS (inducible NO synthase) generates large amounts of •NO over long periods of time in contrast to the controlled •NO generation by eNOS. Hence, expression of iNOS results in impairment of both vasoconstriction and endothelium-dependent vasodilatation, among other effects (Li et al., 2014). Again, studying effects of red (processed) meat consumption on •NO metabolism in *H. pylori* patients would be of interest.

Insulin resistance

As previously mentioned (section *nitric oxide inactivation*), oxidative stress impairs •NO availability. Next to a regulatory function of •NO on the cardiovascular system, •NO also plays a central role in the suppression of insulin resistance on different levels (Kobayashi, 2015). Therefore, oxidative stress following red (processed) meat consumption may lead to impaired •NO availability, through formation of peroxynitrite, and hence contribute to insulin resistance. Previously, a high-fat diet increased pancreatic MDA and lowered pancreatic SOD in rats (Yan et al., 2006). Therefore, diet-induced oxidative stress in the pancreas may also contribute to a disturbed insulin production and secretion by impairment of the insulin-producing β-cells, next to other contributing mechanisms involving oxidative stress (Henriksen et al., 2011). Dietary nitrate (Carlström et al., 2010) and nitrite (Singamsetty et al., 2015) improved features of insulin resistance in different murine models, hence the presence of nitrite in meat products likely does not promote insulin resistance.

Increased insulin resistance was recently reported to be associated with a high intake of red meat and saturated fatty acids from red meat, but not with intake of white meat and saturated fatty acids from white meat. Increased insulin resistance in this study was accompanied with higher ox-LDL (Cocate et al., 2015). Toden et al. (2010) reported unexpectedly increased insulin concentrations in chicken-fed rats, compared to beef-fed rats, despite higher MDA values in the

plasma of beef-fed rats. However, in this study the authors trimmed the fat from the chicken and beef meat, and after cooking, drying and milling, the chicken meat had a lower fat content compared to the beef meat (9% vs. 18% fat). Therefore, they corrected the chicken diet by addition of beef tallow, which is typically highly saturated, and hence, it can be expected that the fatty acid profile was more saturated in the chicken diet. As reviewed by Kennedy et al. (2009), saturated fat also contributes to the development of insulin resistance by various mechanisms among which accumulation of diacylglycerols and ceramides in muscle tissue. In this regard, the intake of saturated fat in meat products may also contribute to the development of insulin resistance. There is a need for controlled intervention studies to disentangle the individual contribution of oxidative stress and saturated fat intake on insulin resistance, in the context of red (processed) meat consumption.

Furthermore, humans infected with *H. pylori* have higher insulin resistance compared to non-infected humans, and this was associated with oxidative stress (Aslan et al., 2006; Gen et al., 2010). Moreover, after eradication of *H. pylori*, insulin resistance was decreased (Gen et al., 2010). Again, *H. pylori* infection could hence worsen or mediate insulin resistance caused by high red (processed) meat consumption.

Oxidative stress in extra-gastrointestinal organs

Diets high in sucrose (Busserolles et al. 2002a; 2002b), high in fat (Matsuzawa-Nagata et al., 2008; Noeman et al., 2011) or combined high sucrose/high fat (Roberts et al., 2000; 2002, 2005) are dietary models that can be used to induce oxidative stress in systemic organs such as kidneys, heart, liver and pancreas. Although red meat consumption has been shown to increase the formation of LOP throughout digestion, a very limited amount of studies investigated the effect of red (processed) meat consumption on systemic oxidative stress parameters. Gorelik et al.

(2013) showed LOP to be absorbed following a red turkey meal in humans. Guéraud et al. (2015) showed that consumption of safflower oil (rich in *n*-6) in combination with haem-Fe resulted in increased hepatic 4-HNE-histidin protein adducts in rats. The oxidation of plasma LDL (Gorelik et al., 2013) and the increased urinary elimination of 4-HNE (Pierre et al., 2004; Chapter V) indicates oxidative stress following red meat consumption is not limited to the gastrointestinal system, but also affects systemic organs. Recently, we showed that beef consumption, in the context of a high sugar diet, promoted oxidative stress in colonic tissues compared to chicken consumption (Chapter V). Moreover, when the beef/sugar diets also contained a high fat content, an TBARS increase in heart and kidneys was observed. These observations might contribute to explaining the epidemiologic association between high processed meat consumption, but not red meat, with coronary heart disease risk (Micha et al., 2010). Renal oxidative stress has been described to contribute to hypertension by inducing renal vasoconstriction and renin release, among other mechanisms (Araujo and Wilcox, 2014). Cardiac oxidative stress may lead to cardiac hypertrophy (Maulik and Kumar, 2012). However, another study showed that *L*-carnitine, a compound abundantly present in beef, protected the heart and liver against oxidation in atherosclerotic rats (Dayanandan et al., 2001). More research is warranted to elucidate the impact of meat consumption and their interaction with other dietary compounds on systemic oxidative stress parameters.

Conclusion

In summary, this review shows that oxidation during digestion is determined by a complex interplay between meat-related factors and dietary compounds. The simultaneous presence of high concentrations of haem-Fe and PUFAs in the diet leads to a high formation of toxic oxidation products during digestion, but this could be effectively prevented when protective

foods are simultaneously ingested. Therefore, the background diet is likely an important factor in the mediation of red (processed) meat associated oxidative stress. Hence, future nutrition studies on the impact of diet on health could focus on dietary patterns rather than on individual foods.

General Discussion and Future Prospects

The epidemiologic association between high red (processed) meat consumption and the risk to develop various chronic diseases is documented widely (e.g. Chan et al., 2011; Micha et al., 2012), but the underlying mechanisms are still a matter of discussion. Many explanatory mechanisms were hypothesized over the years, involving animal protein and fat, HCAs, PAHs, NOCs, LOP and recently, the involvement of TMAO and the sialic acid N-glycolylneuraminic acid (Neu5Gc) (Koeth et al., 2013; Demeyer et al., 2015). The involvement of solely an excess of animal protein and fat is highly unlikely since these nutrients are not specific for red (processed) meat and are also present in poultry, fish and dairy, which are not associated with an increased risk of chronic diseases. Also the formation of HCAs and PAHs during heating of meat cannot fully explain the associations, since other dietary components such as cereals also contribute largely to the dietary intake of total and carcinogenic PAHs (Kazerouni et al., 2001; Falcó et al., 2003; Martí-Cid et al., 2008), while their consumption is not associated with an increased CRC risk. Chicken is a major contributor to HCA (Skog et al., 1997) and PAH intake (Kazerouni et al., 2001), but chicken consumption is not associated with an increased CRC risk in epidemiological studies. Furthermore, Santarelli et al. (2008) argued that the dose of HCA necessary to induce carcinogenic effects in rodents is 1000- to 100 000-fold higher than the doses to which humans are normally exposed by consuming cooked meats. Hence, other pathways are likely involved. During this Ph.D. dissertation, the formation of NOCs and LOP were studied during *in vitro* and *in vivo* digestion of various meat model products. The influence of several meat-related factors on the formation of these compounds was investigated, such as haem-Fe, fat content, nitrite salt and

heating of meat. Furthermore, dietary interactions between red meat and reducing compounds on the formation of LOP were studied *in vitro*.

Stimulating factors in oxidation and nitrosation

The results of Chapters I to III showed that higher contents of haem-Fe and fat, and heating of meat stimulates lipid and protein oxidation in the meat and subsequent digestion. These factors had a similar stimulating effect on the formation of the NOC-derivative DNA adduct O^6-CMG during colonic fermentation. These data are in line with literature, which mainly describes a catalyzing effect of haem-Fe on NOCs (Bingham et al., 2002; Cross et al., 2003; Lewin et al., 2006) and oxidation (Pierre et al., 2004; Pierre et al., 2006; Ijssennagger et al., 2013). The influence of fat content and heating procedures of meat on these processes have been less studied. In agreement to our results, rats consuming cooked meat products had increased fecal TBARS and urinary DHN-MA compared to rats consuming raw meats (Santarelli et al., 2010). During cooking of meat, free Fe^{2+} increases, antioxidant enzymes such as glutathione peroxidase get inactivated (Hoac et al., 2006), and oxymyoglobin releases oxygen, which leads to the production of H_2O_2 (Kanner, 1994). All these reactions stimulate the Fenton reaction through which oxidative processes are initiated. Our results disagree with Sesink et al. (2000) who stated that the association between high red meat consumption and CRC risk is mainly due to its haem-Fe content, and is largely independent of the dietary fat content. Indeed, haem-Fe as a catalyzer of the Fenton reaction is required to stimulate the formation of ROS, but fat as a substrate is required to form LOP to a large extent. It should be noted that Sesink et al. (2000) used hemin-Cl, which is normally not present in the human diet, as a surrogate for haem-Fe. The results of Chapters II and IV showed that the addition of subcutaneous pork fat to meat and subsequent grinding and cooking, increased the formation of LOP during during meat processing and

digestion. In this regard, recent studies also reported on the importance of the fatty acid profile. For example, combination of red meat with fish oil (rich in *n*-3 PUFAs) increases TBARS formation during *in vitro* and *in vivo* digestion, while combination with safflower oil (rich in *n*-6 PUFAs) increases 4-HNE formation (Guéraud et al., 2015; Steppeler et al., 2015; Tirosh et al., 2015). Also, since the precursors ·NO and O_2 preferentially diffuse to a lipid environment, the formation of nitrosating species was described to occur 300 times faster in a lipid compared to an aqueous environment (Liu et al., 1998). This could explain the facilitating role of fat in the formation of NOCs, as observed by the increased formation of O^6-CMG along with higher fat content of the meat (Chapter II). Therefore, the interplay between haem-Fe as a catalyzer and fat as a substrate or medium, likely exerts a central role in the underlying mechanisms between red (processed) meat and the onset of various chronic diseases.

Nitrite curing

In contrast to the hypothesis of Corpet (2011), nitrite-curing of different meat products did not increase the formation of NOC-derivative DNA adducts (O^6-CMG) during *in vitro* colonic fermentation (Chapters I to III). Furthermore, curing with nitrite salt drastically inhibited the formation of oxidation products, however less efficient in meat products with high fat content (20% fat), or very intense heating procedures (30 min at 90°C core temperature). Interestingly, these meat products contained less residual nitrite compared to the other nitrite-cured meat products, in which nitrite had a more efficient antioxidant effect. These results are in accordance to Chenni et al. (2013), who observed an antioxidant effect during the consumption of a 2.5% hemoglobin diet by rats, when nitrite was added at a concentration of 1g/L drinking water, however not significant at lower doses (0.17 g/l nitrite and 0.23 g/l nitrate). However, nitrite could exert both pro- and antioxidant effects depending on its ratio to the present ROS. Darley-

Usmar et al. (1995) reported that a 1:1 ratio of ·NO to ROS enhanced lipid peroxidation through formation of peroxynitrite, while an excess of ·NO resulted in inhibition. Nitrite is present in saliva and the intake of nitrite through processed meat is negligible when compared to the nitrate/nitrite intake through vegetables and fruit. In Belgium, the intake of nitrite through processed meat was estimated at 0.24 mg/day or 6% of the acceptable daily intake (Temme et al., 2011). Therefore, the presence of nitrite salt was not perceived as a risk factor in the present study. Demeyer et al. (2015) suggested that reactive chlorinated intermediates could be formed during the digestion of salted meat products. These chlorinated species such as nitryl-chloride ($Cl\text{-}NO_2$) could arise following the reaction between HOCl and NO_2^- in the stomach, and could form 3-chlorotyrosine and 3-nitrotyrosine following reaction with tyrosine (Eiserich et al., 1996). Hence, it should be kept in mind that digestion of meat products cured with nitrite salt, could contribute to disease through alternative pathways independent of nitrite, which should be investigated in future research.

Microbiota

The fecal inocula of three healthy individuals were used for the *in vitro* colonic fermentation of meat in Chapters I to III. A very large variation was observed in the production of O^6-CMG according to the applied microbiota. This is in agreement with Vanden Bussche et al. (2014) and Engemann et al. (2013), who found large variability in O^6-CMG formation during *in vitro* meat fermentation by human microflora, and in NOC formation out of nitrosamine precursors by cecal porcine microflora respectively. This variation might be crucial, since microbiota are described to have a role in CRC development (Sears et al., 2014). Hereby, one can assume that the microbial composition is the determining factor in toxic colonic NOC production, whereas meat intake could be perceived as a relatively small contributing factor. However, it has been

described that dietary haem-Fe alters the colonic microbial composition, resulting in a higher microbial nitrate reducing capacity (Ijssennagger et al., 2013). Therefore, the effects of red meat on colon health may be mediated by an increased NOC formation through a red meat-associated colonic microflora. In this regard, the formed LOP are likely not primary causal agents involved in the association between red (processed) meat consumption and CRC, since these compounds drastically decrease from the stomach to the colon, as observed in both *in vitro* (Chapters I to III) and *in vivo* trials (Chapter V). However, ROS and oxidation products could contribute to this association, since they can activate NOCs and other genotoxins to its ultimate carcinogenic form, among other mechanisms reviewed in Chapter VI.

Mitigation of oxidation

The results of Chapter IV showed that the formation of toxic oxidation products during the digestion of low-fat and high-fat beef can be mitigated by reducing compounds but, in contrast, can also be stimulated. Hydrophilic reducing compounds such as ascorbic acid and various phenolic acids were able to stimulate oxidation during digestion of the high-fat beef, when added at lower concentrations, likely by reducing the available iron. A similar pro-oxidant effect was reported by Kuffa et al. (2009) who observed a pro-oxidant effect at lower concentrations of grape seed extract during simulated gastric digestion of high-fat (30%) turkey meat, while higher concentrations exerted an antioxidant effect. Other *in vitro* and *in vivo* meat digestion studies usually reported antioxidant effects caused by reducing compounds (Pierre et al., 2003; Verzelloni et al., 2006; Tesoriere et al., 2007; Gorelik et al. 2008; Li et al., 2010; Tagliazucchi et al., 2010; Pierre et al., 2013; Sirota et al., 2013; Gobert et al., 2014). Nowadays, the meat industry aims to produce more 'healthy' meat products by adding natural antioxidants, herbs and/or spices.

Special care must be taken that the addition of these compounds may not result in an unintended higher formation of toxic compounds during digestion.

Systemic oxidative stress

High red (processed) meat consumption is reported to increase the risk for cardiovascular disease and diabetes (Micha et al., 2010; 2012). Chapter V showed that a high consumption of beef in rats may not only increase oxidative stress in the colonic mucosae, where there is a close contact with the digest, but also in in systemic organ systems such as heart and kidney tissues, depending on the background diet. Likely, the high fat and sugar contents of the background diet exerted an important mediating role in the development of the observed beef-induced oxidative stress, with likely modulatory effects of the dietary fatty acid profile. The role of the background diet should therefore be elucidated in future studies, since it could both stimulate or inhibit the formation and activity of toxic compounds. In this regard, red (processed) meat could exert a different impact on health in the context of a 'Western'-like diet which is typically high in fat and sugar, or in the context of a prudent 'Mediterranean'-like or 'Asian'-like diet which are typically high in antioxidants supplied by high amounts of vegetables, fruits, herbs and spices. Oxidation products could play a central role in the development of cardiovascular diseases and diabetes, since oxidation products formed during digestion are absorbed into the bloodstream (Gorelik et al., 2008b; Awada et al., 2012). Among other mechanisms, absorbed oxidation products are able to oxidize LDL (Gorelik et al., 2013) which is considered the first step in developing atherosclerosis, as discussed in Chapter VI.

Alternative hypotheses

The involvement of NOCs and LOP formed during the digestion of red (processed) meat seems likely regarding the onset and/or progression of various chronic diseases. However, additional pathways could contribute to the associations made. Recently, several other explanatory hypotheses were formulated, involving TMAO (Koeth et al., 2013) and the sialic acid *N*-glycolylneuraminic acid (Neu5Gc) (Samraj et al., 2015). Trimethylamine-*N*-oxide is formed through fermentation of *L*-carnitine, which is abundant in red meat, and this compound was hypothesized to accelerate atherosclerosis and hence contribute to the development of coronary heart disease (Koeth et al, 2013). This study has generated much debate and also received much criticism. Supplementation of heart patients with *L*-carnitine attenuated cardiovascular symptoms and reduced mortality in a meta-analysis of 13 controlled trials (DiNicolantonio et al., 2013). Both proatherogenic (Koeth et al., 2013; Tang et al., 2015) and cardioprotective (Johri et al., 2014; Collins et al., 2016) properties of *L*-carnitine and TMAO have been described, which challenges the interpretation of its impact on cardiovascular health. Also of note, plasma TMAO is increased in kidney patients due to its decreased clearance (Missailidis et al, 2016), and kidney patients have increased risk to develop cardiovascular complications (Sarnak, 2003). The TMAO hypothesis can also not explain why only processed meat is associated with an increased risk for cardiovascular disease in epidemiological studies, and not unprocessed red meat (Micha et al, 2012), despite similar or higher concentrations of *L*-carnitine in the latter. If *L*-carnitine would exert protective effects on the cardiovascular system (Johri et al., 2014; Collins et al., 2016), beef could have a dual impact on cardiovascular health with oxidized beef being harmful, but non-oxidized lean beef in the context of a healthy balanced diet being rather protective. Future research should clarify the impact of red (processed) meat in different dietary contexts and conditions since many interacting and dual effects can be expected.

Samraj et al. (2015) reported that the non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc) is particularly present in red meat. The bound form of Neu5Gc is bioavailable and gets incorporated into human tissues. The authors hypothesized that circulating human anti-Neu5Gc antibodies could hence commence inflammation. When human-like Neu5Gc-deficient mice were fed bioavailable Neu5Gc and challenged with anti-Neu5Gc antibodies, they developed evidence of systemic inflammation, and developed a much higher incidence of hepatocellular carcinomas (Samraj et al., 2015). The question remains if this mechanism is also valid to link red meat consumption with CRC.

***Helicobacter* gastritis**

In Chapter VI, we hypothesized that the impact of red (processed) meat consumption on oxidative stress may be synergistic or exacerbated when the consumer is a *H. pylori* gastritis patient or suffers from IBD, since both inflammatory diseases of the gastrointestinal tract may stimulate oxidative stress, both locally and systemically. Since even asymptomatic *H. pylori* infections may be pro-oxidative in the stomach, the interaction between *H. pylori* and meat consumption deserves further research. These synergistic effects remain hypothetical and controlled intervention studies should bring clarification.

Limitations of the presented Ph.D. research

This Ph.D. dissertation faces several limitations that mainly relate to the *in vitro* digestion model that was used and to the laboratory analyses of oxidation and nitrosation products. A static *in vitro* digestion model was used to simulate the gastrointestinal digestion of various model meat products. The application of this static model is rather simple, and allows to digest many meat

samples and hence many different treatments in one incubation run. The ability of digesting many samples in one incubation run has the advantage of excluding inter-incubation variability. However, some physiological aspects of digestion are not represented, such as the absorption of compounds into the bloodstream. E.g. lipid oxidation products will be at least partly absorbed into the bloodstream (Gorelik et al., 2008b; Awada et al., 2012), but also partly pass to the colonic lumen (Chapter V). An absorption step could be included in the model by making use of a dialysis step (e.g. Rodríguez-Roque et al., 2013). The colonic fermentation step could also be simulated in a more physiological relevant way. Many different fermentation models are available, varying from simple batch fermentations to complex multicompartmental continuous systems such as the SHIME, TIM-2 and Lacroix model, as reviewed by Venema and van den Abbeele (2013). Further research could be performed to investigate which bacterial groups are responsible for the formation of NOCs during colon fermentation. More general, the effect of meat intake on the gut microbiome and the interaction with human health deserves more attention in future research, as mentioned above.

We also observed quite some variability in the levels of aldehydes determined by HPLC among experiments, which comprises variability among incubation runs and analytical variability. Two meat treatments (uncured and cured, cooked pork containing 5% fat) were repeated in the first three *in vitro* digestion experiments. The mean values for hexanal concentrations in the duodenal digested uncured pork sample were 1017, 581 and 696 pmol/mL, whereas hexanal in the digested cured pork sample amounted 212, 154, 134 pmol/mL for chapters I, II and III respectively, indicating considerable variability among experiments. In contrast, the mean TBARS values for the duodenal digested uncured pork sample had a higher repeatability, amounting 14.8, 13.3 and 13.6 nmol/mL and for the digested cured pork sample 9.2, 8.4 and 8.2 nmol/mL respectively. Also the mean PCC values were comparable among experiments, amounting respectively 2.81, 2.45 and 2.09 nmol DNPH/mg protein for the digested uncured pork and 1.79, 1.91 and 1.52

nmol DNPH/mg protein for the digested cured pork sample. The amounts of O^6-CMG were also comparable among different incubation runs, and were three times undetected when fermented with microbiota 1, amounted 13.3; 50 ng/mL or was undetected when fermented using microbiota 2, and amounted 504, 486 and 631 ng/mL using microbiota 3, in the respective chapters. Therefore, we can conclude that the *in vitro* digestion model is quite repeatable, but the HPLC method for the analysis of aldehydes could be improved regarding reproducibility, and should be further optimized. For this analysis, the method described by Holley et al. (1993) was applied. This method makes use of a chloroform/methanol extraction. The extracts are subsequently evaporated to dryness and resolved, after which present aldehydes are derivatized with cyclohexanedione. However, it cannot be excluded that some aldehydes were lost during evaporation, as was also mentioned by Holley et al. (1993). Therefore, it could be opted to derivatize the aldehydes in the complete digest, without the chloroform/methanol extraction. From all duplicate incubations of the first three chapters, 87% had a variation coefficient lower than 10% for hexanal values, indicating a high repeatability of replicate incubations in the same run. However, the inter-day precision of this analysis should be further optimized.

The increase in TBARS and PCC during digestion was always distinct, whereas this was sometimes less obvious for the aldehydes measured by HPLC. This observation could be explained by the two following reasons. Firstly, the HPLC method only measures non-bound aldehydes. As previously discussed, 4-HNE can react with proteins involving Michael-addition and formation of Schiff bases (Petersen and Doorn, 2004). Therefore, there was a possible higher 4-HNE proportion bound to proteins during digestion, which hence made them not measurable through the HPLC analysis. Secondly, oxidation may have occurred during storage of the meat samples which were stored at -20°, compared to the digests (-80°C). Therefore, it is likely that 4-

HNE and hexanal measurements were overestimated in the meat samples, and hence oxidation during digestion was underestimated. Furthermore, the absolute quantification of the aldehydes using the HPLC method cannot be guaranteed. When implementing the method in our laboratory, identification and quantification of the peaks were verified by comparing with commercially available standards and by spiking, but we realize that more stringent approaches could be applied. However, the relative effects of haem-Fe, fat content, heating conditions and nitrite-curing were equally observed in the aldehydes as in the TBARS and PCC analyses. Hence, although the validity of the HPLC method may be improved, we feel confident that these issues do not affect the conclusions of this Ph.D.

Since oxidative reactions mainly occur in the proximal digestion system, the applied static *in vitro* model may be considered a valuable tool. As mentioned above, some methodological drawbacks of the model concerning absorption and fermentation should be kept in mind. Some key findings of these initial screening experiments should therefore be confirmed *in vivo*, and could additionally be studied in depth using more complex dynamic *in vitro* digestion models. An additional possible limitation of the present Ph.D. is that the measured NOC-derived DNA adducts in the colonic digests originate from the reaction between NOCs and bacterial DNA during fermentation, and not from the reaction with the added CT-DNA (personal communication). This could be explained due to the highly reactive nature of NOCs. Nevertheless, the measurement of bacterial NOC-derived DNA adducts could be an interesting tool to measure reactive NOCs in colonic digests, but the *in vitro* conclusions should be confirmed using *in vivo* experiments.

Opposing views

Despite the large amount of data showing a relation between high red (processed) meat consumption and the risk for colorectal cancer and various other chronic diseases, a view which is supported by the World Cancer Research Fund (2007), the Superior Health Council of Belgium (2013) and the International Agency for Research on Cancer (Bouvard et al., 2015), some review articles criticize this relationship. The arguments of these papers mainly concern the conclusions of epidemiologic studies (*e.g.* Alexander and Cushing, 2011; Klurfeld, 2015). The Superior Health Council of Belgium (2013) concluded that the convincing power of these review articles suffers from some selectivity in the references used. A series of epidemiologic meta-analyses arguing these associations was published by Alexander et al. (*e.g.* 2010a; 2010b; 2011). This work was founded by the Cattlemen's Beef Board, through the National Cattlemen's Beef Association (NCBA) and the National Pork Board, which could indicate conflicts of interest. In addition, these authors do not consider weak risk factors (less than 1.20) as relevant although these are significant. It must be emphasized that nutritional epidemiologic research generally results in rather low relative risks (RR). The increased RR for colorectal cancer (+17 to 36 % RR per 100g red meat and +18 to 29 % RR per 50g processed meat) (Meta-analyses by Chan et al., 2011; Aune et al., 2013; Xu et al., 2013), coronary heart disease (No increased RR per 100g red meat and +42 % RR per 50g processed meat) (Meta-analyses by Micha et al., 2010; 2012) and diabetes type II (+19% RR per 100g red meat and +51 % RR per 50g processed meat) (Meta-analyses by Micha et al., 2012; Pan et al., 2011) are similar to the magnitude of RRs in other nutritional epidemiologic research. *E.g.* the increased RR for diabetes type II is 20% for the consumption of 330 mL of sugar sweetened soft drinks per day (Meta-analysis by Greenwood et al., 2014) while the RR for coronary heart disease is decreased with 7% per portion of fruit intake (Meta-analysis by Dauchet et al., 2006). It should however also be recognized that these epidemiologic approaches indeed have their limitations and, especially in nutritional research, the presence of

many interacting factors can be expected. We agree on Klurfeld (2015) that these epidemiologic studies show associations but do not prove causality. On the other hand, there is increasing evidence from mechanistic *in vitro* and *in vivo* studies for a causative role for red (processed) meat in this association, although these mechanisms are not completely elucidated (reviewed in Chapter VI). These mechanistic studies were actually poorly discussed by Klurfeld (2015), moreover, not a single study by the expert group of Corpet, Pierre and Guéraud (INRA, Toulouse, France), or any other study concerning the formation and activity of oxidation and nitrosation products were discussed. Some review studies suggest that lean red meat could be beneficial for cardiovascular health (Binnie et al., 2014; McNeill, 2014). There is indeed no evidence that lean red meat would result in a higher cardiovascular risk (Micha et al., 2010; 2012), but such association is present for colorectal cancer (Bouvard et al., 2015; Chan et al., 2011). However, since the formation of toxic compounds during the digestion of meat can be inhibited by many other dietary compounds as shown by several groups (Pierre et al. 2013, Gorelik et al. 2008b, Chapter IV), it seems reasonable to assume that red (processed) meat consumption is not an independent risk factor, but largely depends on other compounds in the background diet as reviewed in Chapter VI.

Final thoughts

The meat industry does efforts to produce more 'healthy' meat products by enrichment of meat products with *n*-3 fatty acids, reduction of the residual nitrite, and addition of pure antioxidants or antioxidant-rich extracts/herbs/spices to meat products. Although *n*-3 fatty acids have nutritional value, special care for the oxidative stability must be considered when these PUFAs are incorporated in haem-Fe rich meat products. Indeed, a high unsaturated fatty acid profile in meat products drastically raises the formation of LOP during digestion, when efficient antioxidants are absent. Consequently, the negative aspects of lipid oxidation may outweigh the

nutritional benefit of *n*-3 fatty acids. Also, the question remains if a further reduction of residual nitrite in processed meats will benefit consumers health, since the balance of •NO:ROS determines the anti- or pro-oxidant outcome of nitrite. The nitrite levels in meat products are negligible compared to nitrate/nitrite intake through vegetables and in the contribution of saliva, concluding that it might not be necessary or desirable to further lower the use of nitrite. Furthermore, addition of sugars or hydrophilic antioxidants such as ascorbic acid to meat products may stimulate oxidation throughout digestion, which should be considered during the formulation of meat products.

In conclusion, the present Ph.D. dissertation showed that oxidative and nitrosative reactions during the digestion of red (processed) meat products, depends on the composition and treatment of the meat, and on their interactions with other dietary compounds during passage in the gastrointestinal tract. The impact of red (processed) meat consumption on health and disease is a challenging and complex field of study, with many interacting factors. More mechanistic and controlled intervention studies are needed to clarify these associations.

Summary

High red (processed) meat consumption is associated with a higher epidemiologic risk to develop various chronic diseases such as colorectal cancer, cardiovascular disease and diabetes. Over the years, several hypotheses explaining these associations have been proposed, but the underlying mechanisms are still a matter of debate. One of the most accepted hypothesis nowadays is the formation of toxic *N*-nitroso-compounds (NOCs) and lipid oxidation products (LOP) during the digestion of red (processed) meat. This Ph.D. dissertation investigated the influence of several meat-related factors on the formation of these compounds during *in vitro* digestion and in a rat feeding trial. More specific, the involvement of haem-Fe (chicken, pork or beef), fat content (1, 5 or 20%), heating conditions (raw, 15 min at core T 65°C or 30 min at core T 90°C) in combination with nitrite curing (0 or 20 g nitrite salt/kg) of meat was studied. Subsequently, it was investigated if oxidation during digestion of lean and high-fat red meat could be prevented by dietary antioxidants. For this purpose, meat model products were subjected to an *in vitro* digestion protocol, consisting of a simulated enzymatic digestion by saliva, gastric juice, duodenal juice and bile juice, followed by colonic fermentation by human microbiota from three healthy individuals. Oxidation throughout digestion was measured by analysis of various LOP such as malondialdehyde (TBARS) (colorimetric), 4-hydroxy-nonenal (4-HNE) and hexanal (HPLC-fluorescence), and protein carbonyl compounds (PCC) (colorimetric) as measurement of protein oxidation. The formation of NOCs was measured by analysis of the NOC-derivative DNA adduct O^6-carboxymethylguanine (O^6-CMG) (U-HPLC-MS/MS).

Oxidation during digestion increased, with a higher formation of LOP, PCC and O^6-CMG when the meat contained higher contents of haem-Fe and fat, and when the meat was subjected to

heating. Nitrite-curing drastically inhibited the formation of LOP and PCC, with no clear effects on O^6-CMG formation. Whereas the concentrations of LOP were maximum following the enzymatic duodenal digestion, O^6-CMG was only formed during the simulated colonic fermentation with a high variation among the three fecal donors. Reducing compounds (ascorbic acid, gallic acid, chlorogenic acid, ferulic acid, caffeic acid, α-tocopherol, quercetin, silibinin) during the digestion of a lean beef product exerted an antioxidant effect to a lower or higher extent (with no effect for α-tocopherol and silibinin). However, reducing compounds had an equivocal effect during the digestion of a high-fat beef product, which is prone to oxidation during digestion. Whereas ascorbic acid and lower concentrations of hydrophilic phenolic acids exerted a pro-oxidant effect, high concentrations of phenolic acids and all doses of lipophilic reducing compounds were antioxidant. These data emphasize that the medium, in which reducing compounds are active, is crucial in determining the pro- or antioxidant outcome of reducing compounds.

Since the combination of red meat and high fat content was shown *in vitro* to stimulate the formation of various LOP, these factors were investigated in a rat feeding study using a full factorial 2 × 2 design. More specifically, rats (n = 6) consumed a diet containing lean chicken, fat chicken, lean beef or fat beef. Following consumption of the experimental diets for 2 weeks, rats on the beef diets had higher TBARS concentrations in the content of the stomach and colon, and in colonic tissues. When the beef meat was consumed in the context of a high dietary fat content, kidney and cardiac TBARS were also increased compared to the chicken diets. Depending on the dietary fat content, the beef diets decreased blood glutathione and increased blood C-reactive protein as a marker of inflammation, and increased urinary excretion of 4-HNE metabolites. This feeding trial showed that the oxidative reactions during red meat digestion are not limited to the gastrointestinal system, but also affect systemic organ systems.

Finally, a literature review was performed in which interactions between meat and other dietary compounds on oxidative processes were studied. Different mechanisms were reviewed, in which oxidative stress following red (processed) meat digestion could contribute to the onset and/or progression of various chronic diseases. In conclusion, oxidation during digestion is determined by a complex interplay between meat-related factors and dietary compounds. The background diet is likely to be an important factor in the mediation of red (processed) meat associated oxidative stress. Hence, future nutrition studies on the impact of diet on health could focus on dietary patterns rather than on individual foods.

Samenvatting

Een hoge consumptie van rood (bewerkt) vlees is geassocieerd met een hoger epidemiologisch risico op de ontwikkeling van verscheidene chronische ziekten zoals colorectale kanker, cardiovasculaire ziekten en diabetes. Door de jaren heen werden verschillende hypotheses geformuleerd om deze associaties te verklaren, maar deze onderliggende mechanismen zijn nog steeds niet volledig opgehelderd. De meest aanvaarde hypothese momenteel is de vorming van toxische *N*-nitroso-verbindingen (NOCs) en lipide oxidatieproducten (LOP) tijdens de gastro-intestinale vertering van rood (bewerkt) vlees. Dit doctoraat onderzocht de invloed van verschillende vlees-gerelateerde factoren op de vorming van deze producten tijdens *in vitro* vertering en een *in vivo* studie in ratten. Meer specifiek werd de invloed bestudeerd van haem-Fe (kippen-, varkens- of rundvlees), vetgehalte (1, 5 of 20% vet), verhitting (rauw, 15 min bij kerntemperatuur van 65°C of 30 min bij kerntemperatuur van 90°C) in combinatie met nitriet pekelen (0 of 20 g nitrietzout/kg). Vervolgens werd onderzocht of oxidatie tijdens de vertering van mager en vet rundvlees voorkomen kan worden door de toevoeging van antioxidanten aan het dieet. Om dit doel te bereiken werden model vleesproducten onderworpen aan een *in vitro* verteringsmodel, bestaande uit een nagebootste vertering door speeksel, maagsap, duodenaalsap en gal, gevolgd door fermentatie door drie individuele humane microbiota in de nagebootste colon. Oxidatie tijdens vertering werd gemeten door analyse van verscheidene LOP zoals malondialdehyde (TBARS) (colorimetrisch), 4-hydroxy-nonenal (4-HNE) en hexanal (HPLC-fluorescentie), en proteïne carbonyl componenten (PCC) (colorimetrisch) als inschatting voor proteïne oxidatie. De vorming van NOCs werd gemeten door analyse van het NOC-geïnduceerde DNA adduct O^6-carboxymethylguanine (O^6-CMG) (U-HPLC-MS/MS).

Oxidatie tijdens vertering nam toe, met een hogere vorming van LOP, PCC en O^6-CMG wanneer het vlees hogere haem-Fe en vetgehaltes bevat, en wanneer het vlees verhit werd. Pekelen met nitrietzout zorgde voor een drastische daling in de vorming van LOP en PCC, met geen duidelijk effect op de vorming van O^6-CMG. Terwijl de LOP concentraties maximaal waren aan het einde van de enzymatisch duodenale vertering, werd O^6-CMG enkel gevormd tijdens de nagebootste colon fermentatie met een hoge variatie tussen de drie fecale donoren. De reducerende verbindingen (ascorbinezuur, galluszuur, chlorogeenzuur, ferulinezuur, cafeïne zuur, α-tocoferol, quercetine, silibinine) hadden een bepaald antioxidant effect tijdens de vertering van het mager rundvlees. Tijdens de vertering van het vet rundvlees, dat gevoelig is voor oxidatie, hadden deze reducerende verbindingen echter een duaal effect. Terwijl ascorbinezuur en lage concentraties van de fenolische zuren een pro-oxidant effect hadden, waren de hogere concentraties van de fenolische zuren en de lipofiele reducerende verbindingen antioxidant. Deze bevindingen benadrukken dat het medium waarin de reducerende verbindingen zich bevinden cruciaal is in de bepaling van de pro- of antioxidant werking van deze verbindingen.

Aangezien de combinatie van rood vlees en hoog vetgehalte de vorming van verschillende LOP stimuleerde tijdens *in vitro* vertering, werden deze factoren onderzocht in een rattenstudie door gebruik te maken van een full factorial 2 × 2 design. Meer specifiek werden ratten (n = 6 voor elke groep) op een hoog vleesdieet gezet; mager kippenvlees, vet kippenvlees, mager rundvlees of vet rundvlees. Nadat deze diëten gedurende 2 weken geconsumeerd werden, hadden ratten op de rundvleesdiëten hogere TBARS concentraties in de maag- en coloninhoud, en in de colonweefsels. Wanneer het rundvlees geconsumeerd werd in de context van een hoog diëtair vetgehalte, waren TBARS in nieren en hart eveneens verhoogd in vergelijking met ratten op de kippendiëten. Afhankelijk van het diëtair vetgehalte, hadden ratten op de rundvleesdiëten

verlaagde concentraties van gluthation en verhoogde concentraties van de inflammatiemerker C-reactief proteïne in het bloed, en verhoogde urinaire excretie van 4-HNE metabolieten. Dit experiment toonde aan dat oxidatieve reacties tijdens de vertering van rood vlees zich niet beperken tot het gastro-intestinaal systeem, maar ook systemische orgaansystemen beïnvloeden.

Tenslotte werd een literatuurstudie uitgevoerd waarin het effect van interacties tussen vlees en andere componenten van het dieet op oxidatieve reacties tijdens vertering werd bestudeerd. Verschillende mechanismen werden besproken, hoe oxidatieve stress volgend op de vertering van rood (bewerkt) vlees kan bijdragen tot de aanvang en/of ontwikkeling van verschillende chronische ziekten. Dit leidt tot de conclusie dat de mate van oxidatie tijdens vertering bepaald wordt door een complexe interactie tussen verschillende vlees-gerelateerde factoren en andere componenten uit het dieet. Het achtergronddieet heeft waarschijnlijk een belangrijke invloed op het al dan niet tot stand komen van oxidatieve stress ten gevolge van een hoge consumptie van rood (bewerkt) vlees. Daarom zouden toekomstige studies omtrent de impact van het dieet op gezondheid zich kunnen focussen op voedingspatronen, in plaats van op individuele voedingscomponenten.

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Curriculum Vitae

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Studies

PhD in Applied Biological Science, *UGent, Belgium (Doctoral examination May the 30th, 2016).*

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Bachelor in Veterinary Medicine, *UGent, Belgium (graduated in 2008).*

High School: Option Mathematics (8 hours) – Science, *St.-Gertrudiscollege, Wetteren (graduated in 2004).*

Research Experience

PhD student (1 March, 2012 – 30 May, 2016): formation of oxidation products and *N*-nitroso-compounds during gastrointestinal digestion of fresh and processed meat – *Department of Animal Production and Food Quality, Faculty of Bioscience Engineering, UGent, Belgium.*

Scientific staff (1 August – 31 December, 2011): mechanisms involved in fatty liver disease in laying hens - *Department of Animal Nutrition, Faculty of Veterinary Science, UGent, Belgium in cooperation with the University of Limpopo, South-Africa.*

Master thesis (August 2010 – June 2011): The copper soil-plant-animal cycle: factors associated with copper status of cattle in the Gilgel Gibe Catchment, Ethiopia - *Department of Animal Nutrition, Faculty of Veterinary Science, UGent, Belgium in cooperation with Jimma University, Ethiopia.*

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Reviewer of

- **Food and Function**

Publications

- **A1 Articles (8)**

Rysman, T., **Van Hecke, T.,** De Smet, S., & Van Royen, G. (2016). Ascorbate and apple phenolics affect protein oxidation in emulsion-type sausages during storage and in vitro digestion. *Journal of Agricultural and Food Chemistry*, in press. DOI: 10.1021/acs.jafc.6b00437. (IF 2014: 2.91; ranking: 2/56 in Agriculture, Multidisciplinary).

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